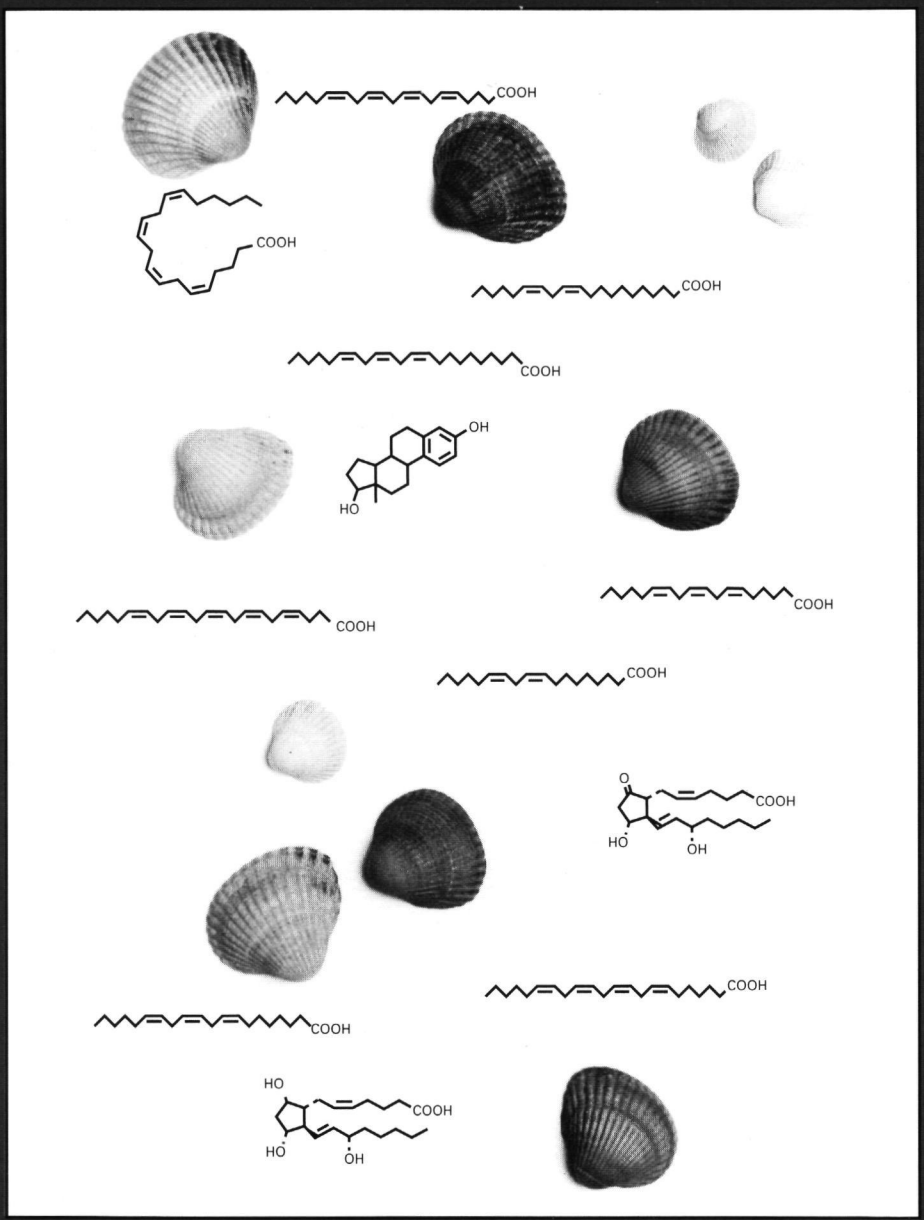
Comparative Studies on Fatty Acid-Binding Proteins

Structure and Binding Properties



Ronald G.H.J. Maatman

COMPARATIVE STUDIES ON FATTY ACID-BINDING PROTEINS

STRUCTURE AND BINDING PROPERTIES

COMPARATIVE STUDIES ON FATTY ACID-BINDING PROTEINS

STRUCTURE AND BINDING PROPERTIES

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Natuurwetenschappen in het bijzonder de Biochemie

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Voor mijn ouders
Voor Mirjam

ABBREVIATIONS

FABP	fatty acid-binding protein
CRABP	cellular retinoic acid-binding protein
CRBP	cellular retinol-binding protein
PAGE	polyacrylamide gel electrophoresis
elisa	enzyme linked immunosorbent assay
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
RACE	rapid amplification of cDNA ends
K_d	dissociation constant
pI	isoelectric point
bp	basepair
kb	kilobases
MMuLV	moloney murine leukaemia virus
Lm	Locusta migratoria
SDS	sodium dodecyl sulphate
IPTG	isopropyl ß-D-thiogalactopyranoside
IgG	immunoglobulin G
DAUDA	11-dansylamino-undecanoic acid
2-AP	2-(9-anthroyloxy)palmitic acid
12-AO	12-(9-anthroyloxy)oleic acid
16-AP	16-(9-anthroyloxy)palmitic acid
POCA	2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Fatty acids are essential for every living cell. They are a source of energy by their oxidation in mitochondria and peroxisomes. Fatty acids are also part of lipids, which form the membrane structures to maintain cell integrity. Various cellular processes are affected by their interactions with enzymes, ion channels, receptors and genes. The transport of fatty acids has to be facilitated by proteins because of their low solubility in aqueous environment. In plasma, fatty acids are transported either bound to albumin or esterified in triacylglycerols of chylomicrons and very-low-density lipoproteins. The uptake across the plasma membrane occurs presumably by diffusion. In cytoplasm of tissues the flux of fatty acids between plasma membrane and cellular organelles is thought to be controlled by the low-molecular mass (15 kDa) fatty acid-binding proteins (FABPs). In the last two decades many reports have been published on the presence of the FABPs in all kinds of tissues of various species. The FABPs were named after the first tissue, they were isolated from. Up to now seven FABP types have definitively been established by the analysis of the amino acid sequence of the isolated protein and/or the cDNA sequence.

In our laboratory studies were devoted to develop adequate methods to assay and isolate FABPs from various tissues of rat, pig and human. Especially the FABPs from liver and heart or skeletal muscle, representing the liver and heart FABP types were investigated. The liver FABP type appeared to be present in liver and intestine, the heart (muscle) FABP type is present in heart, skeletal muscle but also in many other tissues.

In this thesis we devoted especially our attention to the identification and characterization of FABP(s) in human kidney, since this organ presents a marked biochemical heterogeneity. The human kidney is an excretory organ which consists of about 10^6 basic units, called nephrons. Each nephron is composed of a glomerulus, which is a tuft of capillaries interposed between the afferent and the efferent arteriole, and a series of tubules lined by a continuous layer of epithelial cells. The glomeruli are located in the outer part of the kidney, called the cortex, whereas the tubules are present in both the cortex and the inner part of the kidney, the medulla. An ultrafiltrate of plasma formed across the glomerulus passes through the tubules and is modified by reabsorption and secretion. The different tubular segments contribute in different ways to these processes by their different permeability and transport characteristics. In the proximal tubules isosmotic reabsorption of about 70% of filtered water and NaCl takes place based on active sodium transport. Active reabsorption of glucose and amino acids and secretion of organic cations and anions occur in this nephron segment and low-molecular proteins filtered through the glomerulus are reabsorbed and degraded. The proximal tubule forms also the primary target for many nephrotoxic drugs, halogenated hydrocarbons, mycotoxins and heavy metals. The function of the distal tubules and the collecting ducts is the final adjustment of sodium and water excretion. Solute reabsorption in these nephron segments is characterized by finely regulated transport processes against gradients in contrast to the bulk absorption in the proximal tubule.

For the reabsorption and secretion processes the kidney requires much energy which is reflected by the oxygen consumption. Although the kidney represents less than 0.5% of total body mass, renal oxygen consumption constitutes approximately 8% of whole body mass. In general there is a fairly good correlation between the intranephron oxygen consumption and mitochondrial density. The mitochondrial density does not differ much between the various nephron segments in contrast to the metabolism to obtain the required energy. Segmental differences in substrate utilization may reflect differences in cellular uptake and/or mitochondrial transport or differences in activity of the enzymes. The proximal tubules are characterized by aerobic oxidation of substrates and gluconeogenesis whereas the distal tubules show aerobic and anaerobic glycolysis. In the proximal tubules fatty acids serve as energy supply and are metabolized by ß-oxidation in mitochondria and peroxisomes. The excess of fatty acids is incorporated in triacylglycerols. In the distal tubules only mitochondrial oxidation occurs and triacylglycerol synthesis is low.

In view of their different metabolic and transport characteristics the significance of FABP may differ in the various nephron segments. FABP may play a role in the uptake and utilization of fatty acids, but possibly also in the transfer and conversion of other hydrophobic compounds as steroid hermones, prostaglandins, vitamins and drugs. It may also have a protectoryrole against high concentrations of fatty acids or acyl-Coenzyme A esters arising in the case of ischemia or anoxia.

In an additional investigation we describe the primary structure and properties of FABP from the flight muscle of a locust, *Locusta migratoria* for comparison with those of the human heart (muscle) FABP type. The flight muscles of locusts are among the most active muscles known. They display exceptionally high rates of fuel utilization during migratory flight. Fatty acids form then the principal substrate.

AIM AND OUTLINE OF THIS THESIS

In these investigations we tried to elucidate novel structural and functional characteristics of FABPs by biochemical and molecular biological approaches. We focussed on FABP in the kidney, because fatty acids play an important role in the energy supply within this organ and a marked biochemical and physiological intranephron heterogeneity exists.

Chapter 2 gives an overview of the current knowledge of the FABP family. We discussed structural and functional aspects of the various FABP types.

First we studied the presence of FABP in human kidney was studied (Chapter 3). Two FABP types were isolated and characterized. Their physicochemical and binding properties and their distribution within the kidney were investigated.

Subsequently we elucidated their primary structure by analysis of cDNA prepared by reverse transcriptase polymerase chain reaction on human kidney RNA (Chapter 4). This method and immunocytochemistry were also applied to identify and localize the FABP types in rat kidney.

The presence and different localization of two FABP types within the kidney could

relate to differences in binding characteristics. Therefore we expressed both proteins in *Escherichia coli* and performed extensive comparative analysis of binding characteristics of both FABP types with fatty acids of various chain-length and saturation grade and with various other types of hydrophobic ligands (Chapter 5).

In an additional project we performed a comparative analysis of the primary structure of FABP from human muscle and from the flight muscle of *Locusta migratoria* to study evolutionary aspects of this protein (Chapter 6). We isolated the cDNA of flight muscle FABP and manipulated *E.coli* to express the protein. The recombinant protein was compared with recombinant human muscle FABP in radiochemical and fluorescent binding studies.

In the last chapter a summary is given of the results described in this thesis.

CHAPTER 2

INTRODUCTION: STRUCTURAL AND FUNCTIONAL ASPECTS OF CYTOPLASMIC FATTY ACID-BINDING PROTEINS

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Introduction

The solubility and the translocation of hydrophobic ligands is facilitated in intra- and extracellular fluid by so-called lipid-binding or -transfer proteins (1,2). These proteins may catalyze intermembrane transfer and exchange of lipids as fatty acids, retinol, retinoic acid, steroids, phospholipids, but may also modulate the concentration of the free form of the hydrophobic ligand. These proteins may be classified as extra- or intracellular lipid-transfer proteins depending on their location. A special class is formed by the plasma membrane lipid-binding proteins, of which the existence is equivocal. The proteins can be present in lipid-protein complexes (lipoproteins) like in serum, or function as separate lipid carriers. The latter group of proteins may non-covalently bind various types of ligands, but generally they have only high affinity for one class of ligands or to one ligand. Albumin is the main transporter of fatty acids and many other lipophilic ligands in the plasma and the interstitial fluid, but specific binding proteins are present for retinol, vitamin D and steroids (3). Besides albumin, α-fetoprotein and fetuin can function as carriers of fatty acids in fetal plasma (4).

From the capillary to the mitochondrion, fatty acids have to pass through a series of aqueous spaces and membranes (Fig.1). Various models and systems have been suggested for the mechanism of cellular uptake of fatty acids (5,6). The presence of albumin receptors and their involvement in fatty acid uptake by hepatocytes and cardiomyocytes is still a matter of investigation and debate (7-9). A fraction of albumin-fatty acid molecules are transcytosed by endothelial plasmalemmal vesicles into the interstitial fluid (10). Various groups described the presence of plasma membrane proteins that may be involved in fatty acid binding or transmembrane transport, in various cell types as adipocytes, hepatocytes, myocytes, and enterocytes (6,11-15). The molecular mass of the proteins was determined to be about 22, 40-43, or 85 kDa. The first protein was identified by photoaffinity labeling in adipocytes (14), the second was isolated by affinity chromatography from various other cell types (6,12,13), the third was found by binding of radiolabeled sulfosuccinimidyl long-chain fatty acids (15). Antibodies against the 40-43 kDa protein from liver inhibited fatty acid uptake into hepatocytes, adipocytes and cardiac myocytes (12,13,16,17), but by never more than 69%. This indicates that only a portion of the fatty acid uptake may be mediated by a plasma membrane protein.

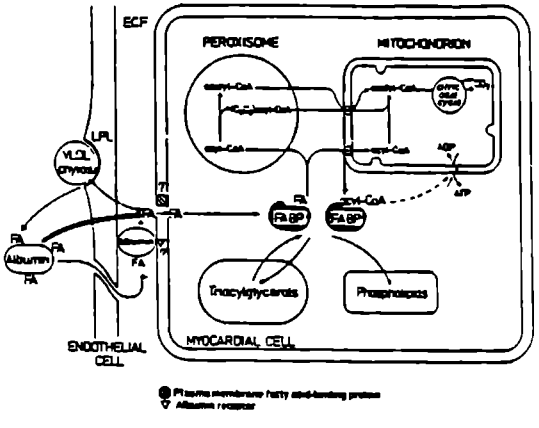

Fig.1 Schematic representation of the way of transport of fatty acids from blood to mitochondrion. Abbreviations: FA, fatty acid; VLDL, very low density lipoproteins; chylos, chylomicrons; LPL, lipoprotein lipase; ECF, extracellular fluid.

The mediated uptake would especially be the case at low fatty acid concentrations, as in the fed condition. Differentiation of adipocytes is accompanied by the expression of a 43-kDa plasma membrane fatty acid-binding protein (17). Recent measurements of fatty acid transport through the adipocyte plasma membrane with fluorescence microscopy indicate that the uptake of a large fraction of long-chain fatty acids is mediated by protein (18).

Reports about the involvement of plasma membrane proteins in fatty acid uptake do not exclude the possibility that the distribution of the ligand into the membrane phospholipids plays an important role. Diffusion through the lipid phase together with equilibration between extracellular albumin and intracellular cytosolic fatty acid-binding proteins (FABPs) may also explain the observed saturation kinetics of the initial rate of fatty acid uptake (19-21). Unionized fatty acids move spontaneously and rapidly across phospholipid bilayers (22). The fast "flip-flop" of uncharged fatty acids across phospholipid bilayers provides a simple and energy-independent mechanism for rapid entry of fatty acids into cells such as adipocytes, hepatocytes and myocytes.

In heart, skeletal muscle and adipose tissue, fatty acids have to pass capillary endothelium prior to uptake into myocytes or adipocytes. Transcytosis of endothelial plasmalemmal vesicles containing albumin-bound fatty acids may play a role (10), but other transcellular transport mechanisms may be more important (23). Cooperative interaction between endothelial and smooth muscle cells (25) and between liver endothelial and parenchymal cells (26) in fatty acid metabolism has been demonstrated. Albumin may play a role in fatty acid transport in the interstitial fluid between different cell types. The presence of plasma membrane fatty acid-binding proteins in endothelial and smooth muscle cells has not been described up to now. Their probable role in transendothelial transport of fatty acids is thus an open issue. Scow and Blanchette-Mackie postulate, on the basis of electron microscopic studies, that fatty acids can be transported across endothelium in an interfacial continuum of membranes composed of the outer (luminal) leaflet of plasma and intracellular membranes of endothelial cells (24). According to their theory, the outer leaflets of the plasma membrane, endoplasmic reticulum and outer mitochondrial membrane form an analogous continuum of cell membranes for fatty acid transport in myocytes. Thus, in this model cytosolic FABPs would not be necessary for uptake and intracellular transport of fatty acids. Nevertheless, this review is devoted to these molecules, although there is a paucity of experimental evidence and FABPs have many other functions, as we will discuss later.

FABPs form a class of cytosolic proteins with a molecular mass of 14-15 kDa, which bind long-chain fatty acids and, depending on type, also other hydrophobic ligands. They have been isolated from or identified in various tissues of mammals, birds and fishes (27), insects (28-30) and worms (31). The different FABP types are denominated by the first tissue of isolation. Generally, these FABPs differ from other cellular lipid-binding proteins as the specific acyl-CoA binding protein (32), phospholipid-transfer proteins (33), glycolipid-transfer proteins (34) and sterol carrier protein (35) by their structure and also by their relative abundance. The cellular retinol-binding proteins (CRBP-I and -II) and retinoic acid-binding

proteins (CRABP-I and -II) belong, on the basis of their primary structure and their conformation to the same superfamily of non-enzymic lipid-binding proteins as the FABPs (36-39). For comparison, some data on these proteins are also given in this survey.

The extracellular lipocalins as ß-lactoglobulin, serum retinol-binding protein, α_1-microglobulin and apolipoprotein D have no sequence similarity with this FABP super-family but show a marked degree of conformational identity (3,40,41). Since the first detection of FABPs in several rat tissues (42,43), several reviews have described the progress of the research on the structure and function of these proteins (5,27,38,39,44-50). Cloning and bacterial expression techniques and biophysical methods have led to new information about the molecular structure and conformation of these proteins and their interaction with the fatty acid ligand (27,51). In this review we will compare and discuss recent data on the structure and function of the different FABP types.

Occurrence of at least 7 FABP types and their cellular and subcellular distribution

FABPs have been isolated from tissues by a variety of procedures, including gel filtration, ion-exchange and affinity chromatography, $(NH_4)_2SO_4$ precipitation and preparative electrophoresis (5). The existence of various tissue-specific FABP molecules was indicated by the necessary adaptations of the isolation procedures for different tissues. Amino acid analysis and sequencing of FABP preparations and cDNA analysis, have established, up to now, the

Table 1 Characteristic differences of members of the FABP family. All differences are derived from human, except ileal FABP and CRBP II. Methionine on position 1 was not taken into consideration for numbering. Positions were indicated after alignment.
*Tyrosine phosphorylation site (15-19): asn-phe-asp-asp-tyr.

Member	Tyr P-site*	Arg "106"	Arg "126"	Tyr "128"	Trp	Cys
Liver FABP	-	thr	122	ser	-	68
Intestinal FABP	-	106	126	phe	6,82	-
Heart FABP	+	106	126	128	8,97	124
Adipocyte FABP	+	106	126	128	8,97	117
Myelin FABP	+	106	126	128	8,97	117,124
Epidermal FABP	+	108	128	130	10,99	119,126
Ileal FABP	-	ala	123	ser	48	68
CRBP I	+	gln	gln	phe	8,88,106,109	95,126
CRBP II	+	gln	gln	phe	8,88,106,109	121,125
CRABP I	-	111	132	132	87,109	95,130
CRABP II	_	111	132	132	87,109	95,130

existence of 7 FABP types (Tables 1 and 2). The occurrence of liver, intestinal and heart FABP types was first described on base of structural and immunochemical data (see refs in 5 and 27), later proved by cDNA sequencing (see 52 and refs in 5 and 27). The large degree of similarity of the heart FABP type with the human and bovine myelin P_2 protein, and the murine adipocyte P_2 (p 422) protein was also noted (27,47,52) and reinforced by analysis of the cDNA sequence of rabbit and human myelin P_2 (53,54), and human adipocyte FABP (55). However, the existence of a specific renal FABP (56,57) was recently disproved (58). In the kidney of mammals both the liver and the heart FABP type are present (58,59). In the male rat kidney, an additional fatty acid-binding α_{2u}-globulin molecule is present, which is not a FABP, but a lipocalin (3). This protein is secreted from the liver and undergoes endocytosis and proteolytic modification in the proximal tubules (57). The occurrence of specific ileal (60-62) and epidermal (63,64) FABP types have recently been established by amino acid and cDNA sequencing. The ileal FABP type was originally isolated as gastrotropin (60), but later identified by others as a cytoplasmic hydrophobic ligand-binding protein (61,62) or a bile acid-binding protein (65-67). More FABP types may exist but have to be carefully identified. Table 1 gives also, for comparison, some characteristics for the CRBPs and CRABPs.

The presence of the FABP types in the tissues given in Table 2 is based on isolation of the protein, on mRNA analysis or on immunohistochemical evidence. Up to now adipose tissue, myelin and epidermis appear to contain one specific FABP type. The immunochemical and/or immunohistochemical evidence for the presence of some FABP types in certain tissues needs to be established. The structure of human muscle FABP is identical to that of human heart FABP (52). The heart FABP type appears to be the most general one in mammalian tissues (68-71).

Table 2 Tissue occurrence of FABP types[1].
[1] The identification of a FABP type in a tissue does not mean its presence in all cell types of that tissue; the FABP type may be limited to specific cells.
[2] Evidence was obtained both by protein and mRNA analysis.

Liver type	liver[2], intestine[2], stomach, kidney[2]
Intestinal type	intestine[2], stomach
Heart type	heart[2], kidney[2], skeletal muscle[2], aorta[2], lung, mammary gland, placenta[2], brain[2], testes, ovary, adrenals[2], stomach
Adipose type	adipose tissue[2]
Myelin type	nervous system[2]
Ileal type	intestine[2], ovary, adrenals, stomach
Epidermal type	skin[2]

Immunocytochemical investigations show in addition to biochemical data, that some tissues express more than one FABP type. Their spatial and cellular distribution, however, differs markedly. After birth, only the liver and intestinal FABP types show a comparable cellular distribution in the intestine, with the same decreasing protein and mRNA gradient from villus top to crypt and from proximal jejunum to colon (65,72-76). The ileal FABP type is primarily expressed in the distal small intestine (65). A heterogenous pattern of cell-specific expression of the liver and intestinal FABP types is present in the intestinal villi of 18-day old rat fetuses (76). The distribution of the liver and heart FABP types in the kidney differs in rat

and man and also between both species (58,59). The liver type is limited to the proximal part of the nephron (man) (59), or present in proximal, distal and collecting tubules (rat) (58). The heart type is mainly (in man) or exclusively (in the rat) present in the distal tubules (58,59,68). Three FABP types (heart, liver, and intestinal) are expressed in the rat stomach, depending on the cell type and developmental stage (77). Recently, the ileal FABP was also demonstrated in the surface mucous cells of the stomach (78). The heart and the ileal FABP type are present in different cells of the ovary (68,78). Discrepancy exists for the presence of the heart FABP type and/or its mRNA in adrenals (68,79) and brain (68,79,80). The FABP type in lung and placenta has also not been established (27). Immunohistochemical observations on rat skeletal muscles show a relation of the distribution of heart FABP type with the muscle fiber type (81). Slow-oxidative and fast-oxidative-glycolytic fibers react strongly with anti-heart FABP antiserum, whereas staining of fast glycolytic fibers is weak or absent (81). Capillary endothelial cells are immunostained in heart and skeletal muscle, exocrine pancreas, digestive tract and thymus (68,81).

The subcellular distribution of FABP has been studied by analysis of isolated cell fractions or by immunoelectron microscopy. Most data sustain the idea, that FABPs are confined to the cytoplasmic matrix outside the membranous organelles. Association with mitochondria, microsomes, peroxisomes, endoplasmic reticulum and lysosomes was not detected by most investigators (see refs in 5,27). With the immunogold technique especially, more locations were observed. The presence of the heart FABP and ileal FABP types were shown in rat heart mitochondria (82) and the nuclear matrix of rat enterocytes (76), respectively. Others observed the occurrence of the heart FABP type in the mitochondria and nuclei of bovine heart by ELISA (83) and isolated FABP from bovine heart mitochondria (84). The FABP from bovine heart mitochondria is identical to one of the two cytosolic isoforms (84). FABP was also found in nuclei of bovine liver (85) and of locust flight muscle (86), and in a slightly higher molecular form in nuclei of rat liver (87,88), but the latter appeared to be histone H_3 (89).

Structural data

The family of FABPs and related proteins contains at least 11 members (Table 1). The primary structure is known for the 7 FABP types, for CRBP-I and -II and CRABP-I and -II. Information was obtained from amino acid sequencing and cDNA analysis. The proteins contain 126-137 amino acids and show various degrees of similarity. An orthologous protein (e.g. heart FABP type) from different mammalian species displays more similarity than two different members (from the same or different tissues) of the same species (27). The aligned amino acid sequence for 6 established FABP types, CRBP-I and CRABP-I and -II from man and CRBP-II from rat is given in Fig.2. The structure of human ileal FABP type and CRBP-II is not known yet. The human heart FABP type has an amino acid sequence similarity to the bovine, rat and mouse orthologue of 99, 89, and 84%, respectively. Its similarity with the human adipocyte, myelin, liver and intestinal FABP types are 65, 61, 27, and 25%, respectively. Its similarity with human CRBP-I (90) and CRABP-I and CRABP-II (91) are 34, 38, and 37%, respectively.

```
          1                                                  50
L-FABP    MS....FSGK YQLQSQENFE AFMKAIGLPE EL..IQKGKD IKGVSEIVQN
I-FABP    MA....FDST WKVDRSENYD KFMEKMGVNI VKRKLAAHDN LK..LTITQE
H-FABP    MVDA..FLGT WKLVDSKNFD DYMKSLGVGF ATRQVASMT. .KPTTIIEKN
My-FABP   .SNK..FLGT WKLVSSENFD DYMKALGVGL ATRKLGNLA. .KPTVIISKK
A-FABP    MCDA..FVGT WKLVSSENFD DYMKEVGVGF ATRKVAGMA. .KPNMIISVN
E-FABP    MATVQQLEGR WRLVDSKGFD EYMKELGVGI ALRKMGAMA. .KPDCIITCD
CRBPI     MPVD..FTGY WKMLVNENFE EYLRALDVNV ALRKIANLL. .KPDKEIVQD
CRBPII    MTKD..QNGT WEMESNENFE GYMKALDIDF ATRKIAVRL. .TQTKIIVQD
CRABPI    MPN...FAGT WKMRSSENFD ELLKALGVNA MLRKVAVAAA SKPHVEIRQD
CRABPII   MPN...FSGN WKIIRSENFE ELLKVLGVNV MLRKIAVAAA SKPAVEIKQE

          51                                                 100
L-FABP    GKHFKFTITA GSKVIQNEFT VGEECE..LE TMTGEKVKTV VQLEGDNKLV
I-FABP    GNKFTVKESS AFRNIEVVFE LGVTFN..YN LADGTELRGT WSLEGNKLI.
H-FABP    GDILTLKTHS TFKNTEISFK LGVEFDE..T TADDRKVKSI VTLDGGKLVH
My-FABP   GDIITTRTES TFKNTEISFK LGQEFEE..T TADNRKTKSI VTLQRGSLNQ
A-FABP    GDVITIKSES TFKNTEISFI LGQEFDE..V TADDRKVKST ITLDGGVLVH
E-FABP    GKNLTIKTES TLKTTQFSCT LGEKFEE..T TADGRKTQTV CNFTDGALVQ
CRBPI     GDHMIIRTLS TFRNYIMDFQ VGKEFEEDLT GIDDRKCMTT VSWDGDKL..
CRBPII    GDNFKTKTNS TFRNYDLDFT VGVEFDEHTK GLDGRNVKTL VTWEGNTLVC
CRABPI    GDQFYIKTST TVRTTEINFK VGEGFEE..E TVDGRKCRSL ATWENENKIH
CRABPII   GDTFYIKTST TVRTTEINFK VGEEFEE..Q TVDGRPCKSL VKWESENKMV

          101                                          144
L-FABP    TTFKNIKSVT E........L NGDIITNTMT LGDIVFKRIS KRI.   127
I-FABP    ..GKFKRTDN GNELNTVREI IGDELVQTYV YEGVEAKRIF KKD.   132
H-FABP    ....LQKWDG QETTLVRELI .DGKLILTLT HGTAVCTRTY EKEA   133
My-FABP   ....VQRWNG KETTIKRKLV .DGKMVAECK MKGVVCTRIY EKV.   131
A-FABP    ....VQKWDG KSTTIKRKRE .DDKLVVECV MKGVTSTRVY ERA.   132
E-FABP    ....HQEWDG KESTITRKLK .DGKLVVECV MNNVTCTRIY EKVE   135
CRBPI     ..QCVQKGEK EGRGWT.QWI EGDELHLEMR VEGVVCKQVF KKVQ   135
CRBPII    ....VQKGEK ENRGWK.QWV EGDKLYLELT CGDQVCRQVF KKK    134
CRABPI    CTQTLLEGDG PKTYWTRELA ND.ELILTFG ADDVVCTRIY VRE.   137
CRABPII   CEQKLLKGEQ PKTSWTRELT NDGELILTMT ADDVVCTRVY VRE.   138
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Fig. 3 Alignment of the liver, intestinal, heart, myelin, adipocyte and epidermal FABP types, and of CRBP I, CRBP II (rat), CRABP I and CRABP II of man. Identical amino acid residues present in at least 5 molecules are shaded.

The main differences are given in Table 1. The alignment shows a marked gap in the C-terminal part of liver FABP.

The finding that the FABP isolated from nurse shark liver is structurally far more closely related to the mammalian FABP types of heart, adipose tissue and myelin than to the mammalian liver FABP type, appears to differ from the conservation of FABP structure observed in mammalian tissues (92). Shark liver is, however, metabolically different from mammalian liver in that it behaves, in some respects, like adipose tissue. The FABPs from flight muscle of two locusts, Schistocerca gregaria and Locusta migratoria, show 41 and 42% identity respectively, with the human heart FABP (93, Maatman et al., unpublished data). A polypeptide in the flat worm, Schistosoma mansoni, displays a comparable degree of similarity with the heart, adipocyte and myelin FABP types of man and less with the liver and intestinal FABP types and the CRBPs (31). Two FABPs from the midgut of the tobacco hornworm, Manduca sexta, show, however, only 20-30% similarity with all FABP types (30).

A common ancestral gene for the FABPs was already proposed in 1983 on the basis of their structural relationship (94). Phylogenetic trees can be constructed on the basis of amino-

acid and/or nonsynonymous nucleotide substitutions (92,95). Chan et al. (95) suggested a divergence of the ancestors of the two major subfamilies of the myelin/adipocyte proteins and the liver/intestinal FABPs before the vertebrate-invertebrate divergence 600 million years ago. The divergence of the members of the heart/myelin/adipocyte FABP family was at some time near the mammal-bird divergence (270 million years; 95). Divergence of the genes for mammalian liver FABP, ileal FABP, intestinal FABP, CRABP-I as well as the progenitor for CRBPs must have occurred prior to the mammal/fish divergence (92).

Tertiary structure and ligand-binding site

Computer analysis and circular dichroism measurements indicate a variable percentage (12-38%) of α-helical structure in various FABP types (5,27). Regions of internal similarity, tandemly arranged repeats, can be detected in different FABPs, but with variations in length, composition and position (5,27).

The availability of recombinant proteins of FABPs in a larger amount and with a higher purity than after isolation from tissues gave a strong impetus to the use of biophysical techniques. Preparations of rat liver (96,97) and intestinal (98,99), murine adipocyte (100,101) and bovine (102) and human (103) heart FABP types have been obtained from Escherichia coli cultures. Studies with fluorescence spectroscopy, nuclear magnetic resonance and X-ray diffraction have given much information about the tertiary structure and/or the lipid-binding site of the FABP types. Photo-affinity labeling and site-directed mutagenesis yield additional information.

Fluorescent fatty acids with bulky side chains show, on the basis of fluorescence enhancement, a strong interaction with the liver FABP type (104-109), but no (or only a weak one) with the heart and adipocyte FABP type (104,109-111). Anthroyloxy-labeled fatty acids were reported to bind to liver, heart and adipocyte FABPs in a bent conformation within a hydrophobic pocket (109,111). The fatty acid bound to liver FABP demonstrates the highest degree of motional constraint, but is present in the most hydrophobic binding site (109,111). Adipocyte and heart FABP types are more similar to each other than either is to the liver FABP type (111). The binding pocket appears to be less open and more hydrophobic, in the rank order: heart FABP, adipocyte FABP, liver FABP (111). The interactions vary with the attachment site of the anthroyloxy group along the length of the fatty acid (105,111).

Cis- and trans-parinaric acids were applied as spectroscopic reporters of the protein-binding site of FABPs (108,112). Tyrosine and tryptophan are present in the binding pocket of liver FABP and intestinal FABP, respectively. The fatty acid-binding site of intestinal FABP is much less polar than that of liver FABP. The liver FABP appears to undergo significant conformational changes upon parinaric acid binding in contrast to intestinal FABP (112).

Recently rat intestinal FABP has been covalently modified with the fluorescent compound Acrylodan at Lys^{27} (113). The shift in fluorescence emission wave length at fatty acid binding provides a direct measure of the concentration of fatty acid bound to the protein. This shift is likely to be due to displacement of the Acrylodan moiety from the hydrophobic fatty

acid binding pocket into the aqueous environment.

^{1}H-NMR investigations also revealed conformational changes of bovine and rat liver FABP upon fatty acid binding (114,115). Sequential ^{1}H resonance assignment obtained by homonuclear twodimensional NMR spectroscopy provided the basis for the elucidation of the secondary structure of bovine heart FABP (116). NMR spectra were obtained for a 1:1 complex of unlabeled palmitate with uniformly ^{13}C-enriched intestinal FABP (51). ^{19}F-NMR spectroscopy on different 6-fluorotryptophan mutant proteins of CRBP-II showed that two tryptophans (Trp^{9} and Trp^{107}) undergo marked changes in their environment upon binding of retinol, while two others (Trp^{89} and Trp^{110}) apparently do not (51,117). ^{19}F NMR analysis of recombinant isotopically labeled proteins appears a sensitive method for monitoring retinoid flux between CRBP-I and CRBP-II (118). The folding of intestinal FABP has been monitored by ^{19}F-NMR after incorporation of 6-fluorotryptophan into the protein (119). The fluorotryptophan substitutions of tryptophan do not markedly change the interaction of CRBPs or intestinal FABP with their ligand (117-119), although minor structural changes occur (120).

^{13}C-carboxyl enriched fatty acids were applied in NMR studies with rat liver and intestinal FABP (121) and porcine ileal FABP (65). The stoichiometry of binding of fatty acid to protein was 1:1 for the intestinal and ileal FABP types, but could vary between 1 and 3 for liver FABP depending on the physical state of the unbound fatty acid (121). The ionization and dissociation behaviour of fatty acids bound to ileal or liver FABP at different pH values was, however, very similar and suggested a binding environment more solvent-accessible than that of intestinal FABP (65,121). NMR samples of porcine ileal FABP with [^{13}C]chenodeoxycholate showed a specific binding interaction, which could be inhibited by fatty acid (65). The carboxylate group of fatty acid bound to intestinal FABP appears to be located in a more interior region and probably involved in ion-pair electrostatic interactions, whereas the carboxylate group(s) of fatty acid(s) bound to liver FABP appear to be present at or near the solvent/protein interface and show no or weak electrostatic interactions (121).

Crystals have been obtained from nearly all members of the FABP family (Table 3). The 3-dimensional structure was shown first for bovine myelin FABP (P_2) (122) and recombinant rat intestinal FABP (123). The structure of the latter molecule was analyzed as holoprotein (123), as apoprotein (124,125) and as protein with bound palmitate (99,126) or oleate (127). Recently, the structures of chicken liver FABP (128), bovine heart FABP (129), recombinant murine adipocyte FABP (130), recombinant human muscle FABP (131) and of an intestinal FABP from Manduca sexta (132) were also determined. Bovine liver FABP (133) and recombinant rat liver FABP (134) have been crystallized, but their tertiary structure is not yet known. The same is the case with ileal FABP (65), CRBP-I (135), CRBP-II (136) and CRABP-I (137).

All these molecules contain 10 anti-parallel ß strands and two short α helices. The ß strands are arranged in two ß-pleated sheets in an orthogonal orientation, giving the protein an overall appearance of a clam. The interior of the protein is extensively solvated. Movement or displacement of some of these ordered water molecules is necessary for fatty acid binding (127,138). The shape of the binding cavity of the intestinal FABP differs from that of human

heart muscle FABP (131,138). It is more elongated, deep inside the protein and allows a slightly bent conformation of the fatty acid. The hydrocarbon tail of the fatty acid bound to heart FABP type has a U-shaped conformation in contrast to the extended form in intestinal FABP (131) (Fig.3). Some of the solvent molecules in the two binding sites are on positions occupied by the fatty acid in the other holoprotein (138). The intestinal, heart, adipocyte, myelin and epidermal FABP types and the CRABPs share the same arginine residues of the binding site (Table 1). These amino acid residues are not present in liver FABP and the CRBPs. The fatty acid-binding site of intestinal FABP, however, also lacks Tyr^{128}. The carboxylate moiety of the fatty acid ligand binds in the same general position in myelin FABP (122) and the FABP from Manduca sexta (132) as in human muscle FABP (131) The structures of the FABPs show marked conformational similarities to the extracellular lipocalins, which lack however 2 ß-strands (40,99).

Table 3 Crystallization of members of the FABP family
* as apoprotein, holoprotein with undefined fatty acid, with palmitic or with oleic acid.

Member	3-D analysis	Resolution (Å)	Reference
Bovine liver FABP			133
Rec. rat intestinal FABP*	+	1.2-2 0	123-127
Bovine nerve myelin FABP	+	2.7	122
Chicken liver FABP	+	2 5	128
Rec. rat liver FABP			134
Rec porcine ileal ILBP			65
Bovine heart FABP	+	3.5	129
Rec. murine adipocyte FABP	+	2 5	101,130
Rec. human muscle FABP	+	2.1	131
Manduca sexta gut FABP	+	1.8	132
Rat liver CRBP I			135
Rat epididymis CRABP I			137
Rec. rat CRBP II			136

All data of biophysical techniques together indicate that although the clam structure is similar for various members of the FABP family, the nature of the internal side-chains is not highly conserved and results in differences of the volume distribution of the cavity, the solvent localization and the conformation of the bound fatty acid, between the

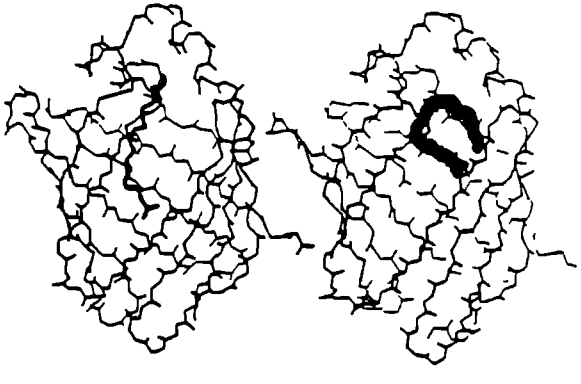

Fig. 4 Comparison of the tertiary structures of rat intestinal FABP (left) and human muscle FABP (right) with bound fatty acid.

different FABP types. Elucidation of the tertiary structure of the liver FABP type and of mutant FABPs will give more information on the interactions between the fatty acid and the different FABP types. The alignment of amino acid sequences of the FABP types (Fig.2) indicates for liver FABP shortening of G and H strands and a wider opening between their two ß-sheets. A unique structure may explain the large differences in ligand specificity, fatty acid stoichiometry and ligand binding site between the liver FABP and the heart and intestinal FABP types.

Table 4 Effect of mutations on binding affinity and thermostability of members of the FABP family.
↓, decrease; ↑, increase; -, no change.

Protein	Mutation	Binding affinity			Stability
		FA	Retinol	RA	
I-FABP	trp-6→tyr				↓
	trp-82→tyr				↓
	arg-106→gln	↓	↑		
H-FABP	arg-106→thr	↓			
CRBP I	gln-109→arg		↓	↑	
CRBP II	gln-109→arg	↑	↓	-	
	gln-129→arg	-	↓	-	
CRABP I	arg-111→gln		-	↓	↑
	arg-131→gln		-	↓	↑
	tyr-133→phe		-		

Some studies have already been performed on mutants of the FABP family (Table 4). Some mutations affect the thermostability of the protein (119,139). Substitution of Arg^{106} in rat intestinal FABP (140) and human heart FABP type [Prinsen and Veerkamp, unpublished data], by glutamine and threonine, respectively markedly decreased the binding affinity for fatty acids and increased that for retinol (140). Glutamine substitution of Arg^{111} or Arg^{131} in CRABP decreased the affinity for retinoic acid (139). The retinol binding affinity was decreased and that for fatty acids or retinoic acid increased by replacement of Gln^{109} by arginine in CRBP-I and CRBP-II (141,142). All these results on mutant proteins underline the importance of electrostatic interactions between lipid and binding protein.

Ligand specificity and stoichiometry

Table 5 gives a survey of the main ligands of the members of the FABP family on base of literature (see refs in 5,27,46,47). The binding of ligands other than fatty acids has not been fully investigated for all FABP types. The heart FABP type will only bind long-chain fatty acids (103,143-146), although binding of acyl-CoA and/or acylcarnitine esters has been reported (143,144). The binding of acyl-CoA to liver FABP (104,145,147) and adipocyte FABP (47) is disputed (146). The liver FABP type appears to be endogeneously associated with fatty acids, but also with heme (148), bilirubin (149) and selenium (150). The liver, intestinal and ileal FABP types isolated from rat intestine contained as endogenous ligands besides fatty acids also butylated hydroxy-toluene and dibutyl- and di(2-ethylhexyl)-esters of phtalic acid (151). Studies

with exogenous ligands showed that a large series of hydrophobic compounds bound to or competed for binding with fatty acids to liver FABP (5,27,104-,147,152-156). These ligands include hexachlorophene, bilirubin, bromosulfophtalein, cholesterol sulfate, oestron sulfate, lysophosphatidylcholine, carcinogens, thyroid hormones, warfarin, a polychlorinated biphenyl derivative, bile salts, some peroxisome proliferators and inhibitors of carnitine palmitoyl transferase, and have generally a lower affinity than fatty acids. A large variation exists in the binding affinity of a series of peroxisomal proliferators in relation with their structure (155, 156). Clofibric acid has little or no affinity in contrast to bezafibrate (104,155-157). Liver FABP and heart FABP type do not bind long-chain alcohols, retinol and retinoic acid. Prostaglandin E_1 (158) and heme (147,154, 157) appear to be bound to rat liver FABP with higher affinity than oleic acid, as are the lipoxygenase metabolites 15-HPETE, 5-HPETE and 5-HETE (159). Other lipoxygenase metabolites including 12-H(P)ETE, 15-HETE and leukotriene (LT)C_4 bind to rat liver FABP to a lesser degree. The cyclooxygenase metabolites PGE_2 and thromboxane B_2 as well as the lipoxygenase metabolite leukotriene B_4 (LTB_4) did not bind to liver FABP (159). The results on prostaglandin binding to liver FABP are equivocal. Cyclopentenone prostaglandines (PGA_1, PGA_2, PGJ_2 and Δ^{12}-PGJ_2) bound more avidly than oleic acid to liver FABP (160). PGD_2, PGE_2 and $PGF_{2\alpha}$ were poor competitors and PGE_1 was intermediate (160). Others found a strong binding of PGE_1 which was not displaced by other prostaglandins (158). Displacement studies could not demonstrate binding of PGE_1 to liver FABP (104,147). Human heart FABP type does not bind prostaglandins or thromboxane B_2 (103,143). Eicosanoid binding to FABPs deserves more attention, especially now recombinant proteins become available.

Table 5 Ligands of members of the FABP family.

Ligand / Member	Fatty acid	Retinol	Retinoic acid	Bile acid	Eicosanoids
Liver FABP	+	-	-	+	+
Intestinal FABP	+	-	-	-	
Heart FABP	+	-	-	-	-
Adipocyte FABP	+	-	+		
Myelin FABP	+	+	+		
Epidermal FABP	+	-	-		
Ileal FABP	+			+	
CRBP	-	+	-		
CRABP	-	-	+		

Liver FABP proved to be identical with a heme-binding protein (161), a selenium-binding protein (150), a lysophosphatidic acid-binding protein (162,163) and a warfarin receptor (153). Bovine myelin FABP also binds retinol and retinoic acid (164). The adipocyte FABP type from man and mouse also binds retinoic acid, but no retinol (55,100,165). Ileal FABP has a higher affinity for chenodeoxycholate than for oleic acid (65).

Liver, heart and intestinal FABP types bind C_{16}-C_{18} saturated and C_{18}-C_{22} poly(unsaturated) fatty acids with comparable affinity (27,97,103,104,143,147). Saturated fatty acids with a

longer chain appear only to be well bound by the liver FABP type (Maatman et al., unpublished data). The apparent dissociation constants of these fatty acids are generally less than 1 μM (5,27). Methodological problems such as aqueous solubility of ligands, aspecific adsorption, and concentration of free ligand impede the interpretation of many investigations of these binding studies (27,47). Recently a theoretical analysis of the interaction of a fluorescently labeled fatty acid with FABP was described as a possible solution for this latter problem (166).

The stoichiometry of binding is one mol fatty acid per mol protein for all FABP types except for the liver FABP type (5,27,167). This FABP type appears to bind 1-3 fatty acids depending on the assay procedures and techniques and the physical state of the applied fatty acid (108,114,121,167-169). Multiple classes of binding sites for fatty acids have been found for this protein (114,169), but not in all cases (108,167). The stoichiometry of fatty acids with bulky side chains is also for liver FABP always 1:1 (104,107,108).

Functions as transfer protein

The main functions ascribed to FABPs are related to the cellular uptake and trafficking of fatty acids to specific cellular compartments or metabolic pools. They may also be involved in the modulation of the intracellular concentration of the fatty acids or their CoA or carnitine esters, and in this way regulate the activity of enzymes involved in lipid metabolism. FABPs may also protect enzymes or membranes from deleterious effects of high fatty acid concentrations. In the case of liver FABP, its capacity in the binding of other ligands may be relevant to the functions.

The role of FABPs in fatty acid uptake and transport was theoretically considered for hepatocytes (19,170) and recently also for cardiomyocytes (171). Indirect evidence on the basis of physiological data and experiments with inhibitors of fatty acid binding to FABP is disputable (27). However, using a photoactivatable, radioiodinated fatty acid analogue, FABP of Hep G2 cells and primary rat hepatocytes and of differentiated 3T3 adipocytes in situ, were labeled in a time- and temperature-dependent fashion (14,172-174). The transfer to and the release of fatty acids from model and natural membranes by FABP has been observed in many studies (107,175-178 and refs in 5 and 27). The transfer does not occur by collision of FABP with membranes, but by aqueous diffusion of the fatty acids (175-177). Only in the case with heart FABP and anthroyloxy-labeled fatty acids a collision model was postulated (110). The rate of transfer of fatty acids from liver FABP to membranes is influenced by aqueous variables, such as pH and ionic strength as well as by the structure of the fatty acid (177). In vitro transfer of fatty acids by FABP was found between two membrane systems separated by a polycarbonate filter (175,178) and between two separated monolayers (175). FABP facilitates also the diffusion of oleic acid in a model cytosol system (179).

The fatty acid exchange rate is much faster on NMR time scale for the intestinal FABP/bilayer than for the liver FABP/bilayer system (121). The transfer rate of fluorescent anthroyloxy-labeled fatty acids from heart FABP to phospholipid bilayers is an order of magnitude greater than the rate of transfer from liver FABP (110,176). The dissociation rates of

the anthroyloxy fatty acids from rat liver FABP (176,177) are in the same order as the dissociation rates of palmitate and oleate from albumin (180). In combination with the comparable dissociation constants of palmitate from albumin (for the three high-affinity sites) and liver FABP, these data suggest that fatty acid uptake in the hepatocyte may be regulated by changes in the level of FABP and the fatty acid/albumin molar ratio. The fatty acid distribution between blood and cytosol may be similar to that of retinol. The distribution of retinol between serum retinol-binding protein and CRBP appears to be at equilibrium and intracellular levels of retinol are regulated by the levels of CRBP (181).

Modulatory functions

FABPs may influence, by supply and modulation of availability of substrate, the activity of enzymes involved in fatty acid and lipid metabolism as mitochondrial and microsomal acyl-CoA synthase and acyl-CoA: glycerol 3-phosphate acyltransferase, acetyl-CoA carboxylase, acyl-CoA: cholesterol acyltransferase, diacylglycerol acyltransferase (see refs in 5,44-46). Some of these results have been related to binding of acyl-CoA by FABP, but acyl-CoA binding protein appears therefore a more real candidate by virtue of its higher affinity for acyl-CoA (146).

FABPs present fatty acids and acyl-CoA to mitochondria and peroxisomes for oxidation (82,175,182,183) and to microsomes for acyl-CoA synthesis (145,178). The use of a holo-FABP as a substrate has not yet been proven. Recently, evidence was obtained for such a role of holo-CRBP in microsomal retinal synthesis (184) and holo-CRABP in retinoic acid metabolism (185).

The binding of different ligands by liver FABP may have various consequences. The sulfation of glycolithocholate by bile salt sulfotransferases was inhibited by liver FABP, in contrast to that of the weaker ligands glycodeoxycholate and glycochenodeoxycholate (186). The presence of heme-binding protein (= FABP) facilitates the heme efflux from rat liver mitochondria (187). The binding of lysophosphatidylcholine (188) and lyso-phosphatidic acid (163) to liver FABP indicate an important role of this protein in regulation of metabolism and utilization of these lysophospholipids in the liver. The involvement of lysophospholipids in signal transduction (189-191) could also be influenced by FABP.

Fatty acids, their CoA and carnitine esters and other lipid mediators, such as eicosanoids and lysophospholipids, influence directly or indirectly various cellular processes by their interaction with enzymes, membranes, ion channels, receptors or genes. Table 6 gives an exemplary survey for the effects of fatty acids. FABPs may have a modulatory role by their influence on the synthesis or the intracellular transport of fatty acids and lipid mediators or by their effect on the availability of the unbound ligand. The availability of the ligand may depend on its affinity to FABP, the presence of competitive ligands and possibly conditions as pH and specific ion concentrations. Fatty acids modulate many steps of the cascade of reactions involved in signal transduction. They may represent modulators, but also first or second

messengers (192). Enzymes of both cyclic AMP and protein kinase C pathways are regulated by fatty acids or in some cases by eicosanoids (192,193).

Arachidonic acid and other fatty acids directly regulate K^+, Ca^{2+} and Cl^- ion channels (194-198), but it is not known whether this is caused by interaction with the ion channel protein or by modulation of its lipid environment (194). Metabolites of arachidonic acid or protein kinase C activity were not involved in these cases, but the former may also stimulate the G-protein of some ion channels in a receptor-independent way (199). Recently, a significant degree of similarity was established between a 131-residue domain of the N-methyl-D-aspartate (NMDA) receptor (residues 263-393 inclusive) and various members of the FABP family (200). Arachidonic acid potentiates the NMDA-receptor current, when glutamate activates the receptor in isolated cerebellar granule cells (201). Arachidonic acid accumulation in pancreatic ß cells may play a role in the signaling process which leads to insulin secretion (202).

Table 6 Effects of fatty acids.

Disturbance of mebrane structure and function
Inhibition of enzymes: Na^+, K^+-ATPase, adenine nucleotide translocase
Modulation of receptor binding: nuclear T_3, angiotensin, glucocorticoid, insulin, opioid
Activation of soluble protein kinase C
Regulation of ion channels: K^+, Ca^{2+}, Cl^-
Modulation of gene expression: FABP, acyl-CoA synthase, stearoyl-CoA desaturase

Receptor-mediated release of unsaturated fatty acids and diacylglycerol contribute synergistically to the activation of protein kinase C (191,203). This activation may form a part of the signal-induced degradation cascade of various membrane phospholipids which is initiated by the action of phospholipase A_2 and phospholipase C. Due to their difference in structure and localization, the isoforms of the protein kinase C family show differences in their sensitivity for fatty acids and other lipid mediators (191). Soluble protein kinase C would be a primary target for unsaturated fatty acids, membrane-associated protein kinase C for diacylglycerols (204).

Fatty acids may interfere with the binding of ligands to their binding proteins, e.g. the binding of retinoic acid to CRABP (205) and the binding of insulin (206), angiotensin (207), or opioids (208) to their receptor. Binding of fatty acid to glutathione S-transferase P noncompetitively inhibited the activity of this enzyme (209).

Fatty acids and/or eicosanoid metabolites amplify the signals from growth factor receptors in mammary epithelial cells (210). Fatty acids can interact with nuclear or cytoplasmic steroid or thyroid hormone receptors to modulate positively or negatively the binding of these hormones (211-216). They can also influence in this way the transfer of steroid or thyroid hormone information from one binding protein to another. Fatty acylation of proteins has also been suggested as an event involved in second messenger action (217). FABP may modulate all these actions of fatty acids.

A protective role of FABP in ischemia of tissues as heart, intestine, kidney and liver appears also probable. The intracellular rise of fatty acid concentration at ischemia can perturb functions of enzymes, membranes and ion channels (218-222) and in this way e.g. interfere with heart function (23).

A phosphorylated form of adipocyte FABP may be an intermediate in the insulin signaling pathway (223-225). This form accumulates, though in a very low percentage of total FABP, in adipocytes in the presence of insulin or vanadate and phenylarsine oxide, an inhibitor of tyrosine phosphatases (226). In vitro, adipocyte FABP can be phosphorylated on Tyr^{19} by the insulin receptor tyrosine kinase with a positive effect of the presence of a bound fatty acid (100,227,228). The presence of a bound fatty acid on adipocyte FABP increases the affinity of the insulin receptor for this FABP (228). The same phosphorylation site for tyrosine kinases is apparently present in heart, myelin and epidermal FABP, and in CRBP-I and -II (Table 1). A slight amount of the phosphorylated form of the heart FABP type (< 1% of total FABP) could be isolated from insulin-stimulated cardiomyocytes (229), but not from insulin-stimulated rat soleus muscle or cultured rat muscle cells (Prinsen and Veerkamp, unpublished data).

Recently, evidence was obtained for the involvement of fatty acids in gene regulation (230-236) as was earlier established for retinoids. A role for FABPs as trans factors in the expression of lipogenic enzymes was suggested by Clarke and Armstrong (48). In fully differentiated adipocytes a high level of adipocyte FABP gene transcription can be supported by fatty acid supply (230,231). Long-chain fatty acids are able to activate, in preadipose cells, the expression of the genes encoding adipocyte FABP and acyl-CoA synthetase (232,233). Fatty acids dramatically activate the expression of mRNA for adipocyte FABP and the Fos-related gene Fra1 in 3T3 preadipocytes, without initiating the general program of adipocyte differentiation (234). The process of activation of the FABP gene by fatty acids was not found in non-adipose cells (233,234). Activation of the genes of adipocyte FABP and acyl-CoA synthetase by fatty acids does not appear to be mediated by the CCAAT enhancer binding protein (233). The increase of the steady state levels of adipocyte FABP mRNA in preadipocytes is likely to be the result of post-transcriptional mechanisms, most likely, message stabilization (234). Induction of adipocyte FABP in preadipocytes appears to be exerted by unprocessed fatty acid (232). Recent findings implicate arachidonic acid as an important inhibitory regulator of stearoyl-CoA desaturase gene expression in lymphoid cells (235). Selected fatty acids modulate gene expression in human breast cancer cells (236).

The involvement of FABPs in growth and cell replication is suggested by various investigations. The level of liver FABP (237,238) and the activity of the liver FABP promoter (239) were markedly enhanced in normal hepatocytes during mitosis. Linoleic acid specifically stimulated the growth and DNA synthesis of hepatoma cell clones that were stably transfected with the coding sequence of liver FABP and expressed this protein (240). Metabolism of linoleic acid to oxygenated derivatives was apparently necessary. Growth stimulatory and growth inhibitory eicosanoids bind well to liver FABP and even with higher affinity than fatty acids (159,160). Liver FABP is the principal early target protein of the reactive metabolites of the carcinogen N-(2-fluorenyl)acetamide (241).

An isoform of bovine heart FABP was originally isolated from bovine lactating mammary gland (242). This compound was named mammary-derived growth inhibitor since it had inhibitory action on growth of various normal and transformed mammary epithelial cell

lines. Its expression is highest in lactating mammary gland (243). Bovine heart FABP had no inhibitory effect on cell proliferation. The heart FABP type was also isolated from, and identified in, mammary gland of lactating rat and mouse (244,245).

Gene structure and regulation of expression

Screening of genomic libraries with specific cDNAs revealed the genes for nine members of the FABP family: adipocyte, myelin, heart, intestinal and liver FABP types, CRBP I and II and CRABP I and II. Only the genes for epidermal and ileal FABP types have not been identified up to now. All genes except the intestinal and heart FABP and the CRABP II genes were identified in one species (Table 7). The intestinal FABP gene was elucidated in mouse (246), rat (247) and human (247), the heart FABP gene in mouse and cattle (248,249) and the CRABP II gene in mouse (250) and human (251). Most of the genes were however detected in other species by low stringency Southern hybridization. The overall organization of the genes is identical, four exons and three introns (Fig.4). The exon/intron boundaries are similar in all genes but the length of the intron sequences varies markedly. The CRBP genes have relatively large introns.

Table 7 Chromosomal location of genes of lipid-binding proteins of the FABP family.

	Chromosome (region)	
Protein	Human	Mouse
Adipocyte FABP	?	3
Heart FABP	1	4,8 and 10 or 15
Intestinal FABP	4 (q28-q31)	3
Liver FABP	2 (p12-q11)	6
CRBP I	3 (q21-q22)	9
CRBP II	3	9
CRABP I	15	9
CRABP II	2 or 11 ?	2

Using somatic cell-hybrids and restriction fragment length polymorphisms the chromosomal localization of most of these genes was determined in human and mouse (Table 8). The last few years detailed studies on the 5' promoter region revealed much information about the regulatory elements for the tissue-specific expression of these genes. We will separately discuss the structure and regulation of the expression of the different genes.

Table 8 Identified genes of the FABP family.

	Species			
Member	Mouse	Rat	Bovine	Human
Adipocyte FABP	+			
Myelin FABP	+			
Heart FABP	+		+	
Intestinal FABP	+	+		+
Liver FABP		+		
CRBP I				+
CRBP II		+		
CRABP I	+			
CRABP II	+			+

Adipocyte FABP gene

The mouse adipocyte FABP or aP_2 gene has a total length of 4.2 kb containing 4 exons encoding 25, 57, 34 and 16 amino acids, respectively separated by 3 introns of 2.2, 0.5 and 0.7

kb (252,253) (Fig. 4). The mouse gene is localized on chromosome 3 within 2.2 cM of the carbonic anhydrase 2 locus (79).

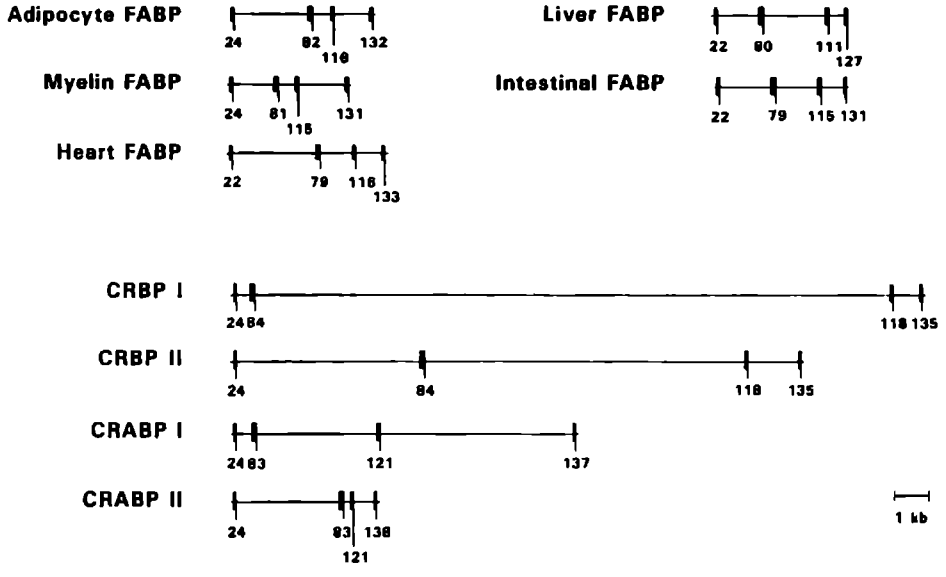

Fig. 4 Comparison of the structures of FABP genes. The exon/intron structures of the mouse adipocyte, myelin, intestinal FABP, CRABP I and II, rat liver FABP, CRBP II, bovine heart FABP and human CRBP I genes. The locations of the splice sites are indicated by the number of the amino acid.

Most studies on the regulation of adipocyte FABP gene expression have been performed with 3T3, Ob 1771 and BCFC-1 cell lines. All cells exhibit, as preadipocytes, the properties of growing fibroblasts. When the confluent cells are treated with appropriate media and hormones, growth factors or pharmacologic agents they undergo adipocyte differentiation (254-256). Differentiation can be induced by various combinations of dexamethasone, insulin and methylisobutylxanthine (257). Addition of retinoic acid, together with these compounds, to preadipocytes inhibited differentiation (257). De novo synthesis of fatty acids and triacylglycerol formation increase at differentiation and the cells accumulate large lipid droplets. Activities of enzymes involved in lipogenesis, lipolysis and glycolysis increase due to increased synthesis. Levels of other proteins, e.g. cytoskeletal proteins decrease. Examples of enzymes which increase in activity 10- to 100-fold are glycerol 3-phosphate dehydrogenase, fatty acid synthase, glycerol 3-phosphate acyltransferase, malic enzyme, lipoprotein lipase, stearoyl-CoA desaturase and also serine protease (adipsin) (255). Also the content of acyl-CoA binding protein (258), adipocyte FABP (259,260) and a 88 kDa membrane protein implicated in fatty acid-binding (261) increase markedly. The genes of these differentiation-dependent proteins have been shown to be regulated by various adipogenic and antiadipogenic factors. A number of cis-acting regulatory elements within the promoters of these genes have been identified together with various trans-acting nuclear factors, that activate or derepress transcription (255,262). We will discuss in the

following especially those, which influence the expression of the adipocyte FABP gene. All cis-regulatory elements in the 5' flanking region of the adipocyte FABP gene and their possible trans-acting factors are depicted in Fig. 5.

Hunt et al. (253) compared the 5' promoter region of genes of which expression is induced upon adipocyte differentiation, the glycerol 3-phosphate dehydrogenase, the serine protease (adipsin) and the adipocyte FABP gene, and looked for cis-regulatory sequences. A fat-specific element of 13 bp with a consensus sequence 5'-GGCT/ACTG-GTCAG/TG-3', called FSE1, was found in multiple copies in all three genes. An additional element, FSE2, 5'-ACTC-/GAGAG-GAAAAG-3', was only found in the adipocyte FABP and glycerol 3-phosphate dehydrogenase genes (253). The first 168 bp of the 5' flanking sequence of the adipocyte FABP gene appeared to be sufficient to drive differentiation-dependent expression. Within this sequence a 28 bp element is located which inhibits expression in preadipocytes and binds a fos-like protein (263). Fusion products of 858 or 248 bp of the 5' flanking sequence of the adipocyte FABP gene with the bacterial chloramphenicol acetyltransferase (CAT) gene were transfected into preadipocytes. Differentiation induced by adipogenic agents revealed only expression of the longer construct with dexamethasone, which indicates the presence of a glucocorticoid-regulatory element. Expression of both constructs was induced by 8-bromoadenosine 3',5'-cyclic monophosphate which suggests a response element for cAMP in the first 248 bp (264). Deletion analysis of the short construct established the presence of a sequence containing an overlapping positive and negative regulatory element. The positive regulatory element contains a consensus activator protein 1 (AP-1) binding sequence (265) which is activated by the trans-activating fos and jun proteins (263,266). The inhibitory effect of the negative regulatory element in confluent preadipocytes is relieved by cAMP (265).

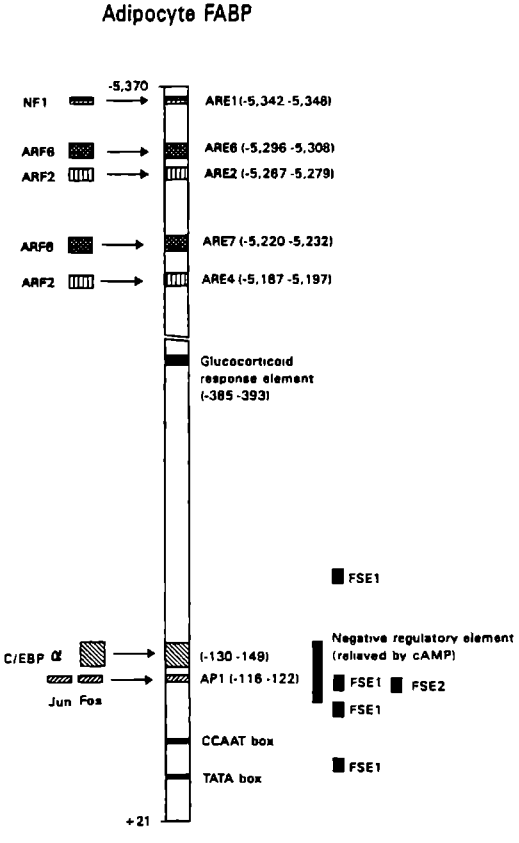

Fig. 5 Cis-acting elements and trans-acting factors in the 5' flanking region of the adipocyte FABP gene.

A binding sequence for the trans-activating CCAAT/enhancer binding protein (C/EBP) is located directly upstream of the AP-1 sequence, within the negative regulatory element. This sequence is also present in the promoter region of the differentiation-induced stearoyl-CoA

desaturase 1 gene (267). The role of AP-1 and C/EBP was analyzed by deletions and point mutations in their binding sequences. These sequences appear to regulate gene expression during adipocyte differentiation (268). In a cell-free transcription system it was also shown that the expression driven by promoters of adipocyte differentiation-induced genes is activated by C/EBP (269). Antibodies against C/EBP and oligonucleotides with the C/EBP consensus binding sequence drastically decreased this in vitro transcription. Additional evidence for the role of C/EBP in adipocyte differentiation was demonstrated by transfecting preadipocytes with a vector expressing antisense C/EBP RNA (270). The cells did not show C/EBP expression on RNA and protein level and no cytoplasmic triglyceride accumulation. The C/EBP region and adjacent sites also regulate A-FABP expression in human adipocytes (271). Cao et al. (272) showed the existence of three C/EBP isoforms which are all regulated during differentiation. These trans-activating factors and their way of interaction with cis-acting elements in the upstream region of the adipocyte FABP gene were recently reviewed (262).

The far upstream region of the 5' flanking region of the adipocyte FABP gene appeared to have a adipocyte-specific enhancer (273). Transgenic mice containing 168 bp, 247 bp, 1.7 kb or 5.4 kb of 5' flanking sequence of the adipocyte FABP gene linked to the CAT gene did not show high levels of chloramphenicol transferase in their adipose tissue until the extension amounted to 5.4 kb (273). More detailed analysis of this enhancer revealed five cis-acting adipocyte regulatory elements designated ARE1, ARE2, ARE4, ARE6 and ARE7 (274,275). The ARE1 site binds a member of the nuclear factor-1 (NF-1) family. Multimers of this site have no activity on their own; nevertheless, when this site is mutated, there is a significant reduction in enhancer activity. Multimers of ARE2 and ARE4 stimulate promoter acitvity in several cell types. These elements share a common motif and appear to be recognized by adipocyte regulatory factor 2 (ARF2). By contrast, multimers of ARE6 and ARE7 stimulate promoter activity only in adipocytes. These elements also share a common motif which is bound by adipocyte regulatory factor 6 (ARF6). The binding of this factor to both elements is differentiation dependent. Mutational analysis established that ARE6 is critical for enhancer activity.

The differentiation from preadipocytes to adipocytes is controlled by several factors which also affect the expression of adipocyte FABP. Insulin growth factor-1 (IGF-1) and in higher concentrations also insulin are able to induce preadipocyte differentiation (276). Both increase the mRNA expression level of the fatty acid synthase and the adipocyte FABP gene. Pioglitazone, an antidiabetic drug showed a similar effect on the expression of adipocyte FABP (277). It was suggested that this agent sensitizes the 3T3-L1 cells for insulin. Incubation of fully differentiated 3T3-L1 adipocytes with tumor necrosis factor-α (TNF-α) resulted in an insulin resistance (278). The expression of differentiation-induced genes encoding glucose transporters 1 and 4 (GLUT1 and 4), C/EBP and proteins involved in fatty acid metabolism (stearoyl CoA desaturase, acyl-CoA synthase and adipocyte FABP) was completely or markedly repressed. The effect is probably due to the lower expression of C/EBP which controls the expression of the other genes (279). Transforming growth factor-ß, TNF-α and retinoic acid increase the

expression of mRNA of transcription factors c-fos, jun-B and c-jun, but inhibit C/EBP mRNA expression (280). Both the increase and the inhibition may cause the attenuation of differentiation of preadipocytes.

Since preadipocytes and adipocytes are exposed to changing levels of fatty acids these substrates may regulate the expression of genes during adipogenesis. Oleate addition to confluent preadipocytes potentiated the effect of dexamethasone in inducing expression of adipocyte FABP mRNA (230,233). After differentiation, the level of expression in adipocytes could be maintained by fatty acids (230,231). Especially long-chain fatty acids are able to activate adipocyte FABP gene expression (232-234). In fully differentiated adipocytes impairment of fatty acid synthesis by glucose deprivation leads to an inhibition of expression of the genes of adipocyte FABP, adipsin and glycerol 3-phosphate dehydrogenase (231). Supplementation of the culture medium with fatty acids prevents the decrease of adipocyte FABP gene expression, whereas the expression of adipsin and glycerol 3-phosphate dehydrogenase is unaffected (231). The activation of FABP gene expression by dexamethasone and fatty acids in 3T3 preadipocytes does not appear to be mediated by C/EBP, since C/EBP is not expressed (233). Besides the effect on the adipocyte FABP gene, fatty acids also induce the expression of the acyl-CoA synthase gene and the Fos-related Fra-1 gene in preadipocytes (234). The mechanism by which fatty acids induce gene expression is unclear but seems to differ from C/EBP mediated expression during adipogenesis (233).

Myelin FABP gene

The mouse myelin FABP or myelin P2 gene with a length of 3.2 kb contains four exons encoding 25, 57, 34 and 16 amino acids, respectively separated by 3 introns of 1.2, 0.2 and 1.3 kb in length (281)(Fig.4).

Myelin P_2 protein is one of the major proteins of peripheral nervous system myelin besides the P_0 glycoprotein and myelin basic protein (53,54). It is not detected in central nervous system myelin.

In the 5' flanking region two AP-1 binding sites are present besides a candidate TATA element and 2 CAAT sequences. A band shift of labeled myelin FABP promoter DNA was seen after incubation with nuclear extracts containing c-jun. These results suggest that c-jun may regulate expression of myelin FABP (281).

Heart FABP gene

The heart FABP gene was detected in the chicken, rat, mouse and human DNA using the rat heart FABP cDNA as a probe in Southern blot analysis (79). Three loci in the mouse genome located on different chromosomes (4, 8 and 10 or 12) reacted with this probe under stringent conditions. The loci may represent pseudogenes and/or genes encoding proteins with a high similarity to the heart FABP. Two FABP isoforms were found in bovine heart (84). Two bovine heart FABP mRNAs with the codon for Asn or Asp at position 98, respectively were isolated, which may derive from different genes (282). Recently the bovine heart FABP gene

has been isolated and sequenced (248). It consists of four exons encoding 22, 57, 37 and 17 amino acids respectively separated by 3 introns of 2.5, 1.1 and 0.8 kb (Fig. 4). Also the mouse heart FABP gene has been identified, including some part of its 5' flanking region (249). The mouse heart FABP gene is located on chromosome 4.

At fetal age from day 16 on heart FABP is present at a low level in rat heart, but after birth it increases in rat heart and skeletal muscle up to 70 days (70, 283-285). Heart FABP mRNA levels rise rapidly in rat heart during the 2 days prior to and after birth, reaching peak levels by the early weaning period (79). Immunohistochemical studies on the ontogeny of heart FABP type demonstrated a more extensive tissue distribution and earlier ontogenic expression in the rat (68) than might be expected from mRNA analyses. Rat kidney shows a postnatal increase of the content of the heart FABP type (283). Heart FABP mRNA showed in this tissue biphasic alterations with peaks in late gestation and at 8 days of postnatal age (79). Rat brain and testis show an increase of heart FABP mRNA after weaning (79). A rapid increase in heart FABP mRNA was also observed in the late gestation placenta (79).

The main FABP present in mammary gland of rat, mouse and cattle appears to be the heart FABP type (244,245,287,288). A growth inhibitor isolated from bovine mammary gland (MDGI) appeared to be an isoform of heart FABP (242). The expression of the protein is highest in terminally differentiated lactating mammary gland and absent in virgin mammary gland (243,287,288). C-terminal amino acids seem to carry the signal for inhibition of tumor cell proliferation (289). A MDGI form at nine amino acid positions not identical with heart FABP was detected by screening of a cDNA library derived from the mammary gland of a pregnant mouse (290). Also the rat mammary gland contains an additional FABP, which is presumably an isoform of heart FABP (244). MDGI may be a protein in low abundance and/or localized to a specific group of mammary epithelial cells.

In brain the main FABP type appears also to be the heart type (69-71), although both the presence (79) and absence (80) of heart FABP mRNA were reported. The presence of more FABP types in bovine brain was suggested on base of amino acid sequence heterogeneity of isolated preparations (291,292). Under relatively relaxed stringency employed for primer extension experiments two products were found with brain mRNA in contrast to with heart, muscle and kidney mRNA (79). Recently a mouse gene coding for a new type of FABP was identified by a homology-based PCR approach (293). Using mRNA from brain as template its DNA sequence was confirmed by reverse transcriptase PCR. On base of the localisation of the transcript and the protein in the olfactory nerve layer, the new FABP is called olfactory FABP. It has 88% similarity with heart FABP type and 70% with adipocyte and myelin FABP types.

Epidermal FABP gene

The gene for the epidermal FABP type (63,64) has not yet been identified. Expression levels of this FABP are low in normal epidermis, higher in cultured keratinocytes and still higher in psoriatic skin (63,64). Krieg et al. (294) described the cDNA for a mal-1 protein, a keratinocyte lipid-binding protein, which is up-regulated in both benign papilloma and malignant

squamous cell carcinomas stages during tumor development. The derived amino acid sequence is most identical with the mouse myelin FABP and adipocyte FABP types. In Southern blot analysis the gene was detected in DNA from mouse epidermis and from papillomas and squamous cell carcinomas. It was suggested that more copies of the gene were present in the mouse genome. An additional band was observed in DNA from tumors indicating rearrangements or changes in the methylation pattern of the mal-1 gene.

Intestinal FABP gene

The complete intestinal FABP gene was elucidated for human and mouse (246,247). For the rat intestinal FABP gene only the first two exons, the first intron and 11 kb of 5' flanking sequence were isolated (247). The human and mouse intestinal FABP gene consist of four exons encoding 23, 57, 36 and 16 amino acids, respectively separated by 3 introns of 1.2, 1.0-1.8 and 0.4 kb (Fig. 4). In the second intron of the human gene a trinucleotide repeat of $(TTA)_{13}$ is present (247,295). Using the rat intestinal FABP cDNA as a probe a restriction fragment length polymorphism was demonstrated after Taq I digestion of genomic DNA from various cattle species (296). The gene is located on human chromosome 4 in the q28-q31 region and on mouse chromosome 3 between the amylase 1,2 and alcohol dehydrogenase 3 loci (246,247) (Table 8).

The regulatory elements of the 5' flanking region of the intestinal FABP gene are depicted in Fig. 6. Alignment of the 5' flanking sequences of the mouse, rat and human intestinal FABP genes revealed the presence of three conserved domains and of more copies of a 14 bp consensus sequence (5'-TGAACTTTGAACTT). The positioning of these sequence elements is well conserved among the intestinal FABP genes of mouse, rat and human despite the difference in the relative position of elements I, II and III. In the mouse and rat genes three copies are present. The human intestinal FABP gene is missing 180 bp between the conserved domains I and II, but has an additional partial fourth copy at a position analogous to the most 5' 14-bp element of the rat and mouse genes (246). This consensus sequence or parts of it are also found in the 5' flanking region of the CRBP II and the apolipoprotein A-I gene (247,297). These genes are expressed in the small intestine like the intestinal FABP gene. Only the rat intestinal FABP gene contains a B2 repetitive element. All intestinal FABP genes have the transcription-regulatory CCAAT and TATA box sequences (246,247). Several cAMP-reponsive elements are also present. The latter contains a 24 bp element that binds nuclear factors which are present in colon but not in small intestine epithelial cells. Directly downstream this element the consensus sequence for C/EBPα is located. Between nucleotides -103 to +28 one copy of the 14 bp element is present. This element binds two transcription factors, hepatic nuclear factor4 (HNF4) and apolipoprotein regulatory protein-1 (ARP-1), both belonging to the steroid hormone receptor superfamily (298).

Study of the regulation of the intestinal FABP gene is of large interest. This gene is only expressed in the enterocytes in mouse, rat and human (246,299,300), one of the four principal epithelial cell types originating from the multipotent stem cell in the base of the intestinal crypt.

Expression is activated during their translocation from the upper crypt to the base of the intestinal villi and continues during migration to the apical extrusion zone. In this way a crypt-to-villus (vertical) gradient of intestinal FABP and intestinal FABP mRNA of mouse, rat and human is formed (72,247,299-301) The level of the protein and its mRNA vary also along the cephalo-caudal (horizontal) axis of the gut. The highest concentrations are present in the jejunum and ileum of mouse and rat (65,72,76,299,300). It is also present in the proximal colon, but absent from stomach and distal colon.

The mouse intestinal FABP gene is activated in the gut epithelium between embryonic days 15 and 16 (246). In the rat the intestinal FABP mRNA was first detectable in small intestine and between the 19th and 21st day of gestation (286). The rat enterocytes of jejunum and ileum do not demonstrate uniform staining for intestinal FABP until postnatal day 1 (76). This mosaic cellular pattern starts in the proximal small intestine by fetal day 18 and occurs 1-2 days later in distal small intestine. In the mouse a mosaic pattern of intestinal FABP is observed up to the fourth postnatal day only in cells located in the intervillus region, but not in the villus-associated enterocytes (246). In adult animals the intestinal FABP gene is activated in mouse and rat enterocytes after they complete passage through the cell cycle and exit the crypt (246). The cell-specific and regional differences of intestinal FABP evident at the early life are maintained throughout adulthood in rat and mouse (246,299).

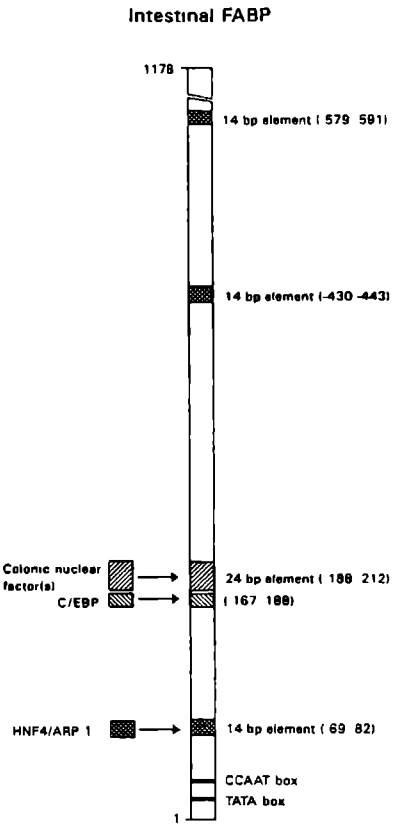

Fig. 6 Cis-acting elements and trans-acting factors in the 5' flanking region of the intestinal FABP gene.

Gordon and his associates have used transgenic mice containing fusion genes of the 5' nontranscribed domain of intestinal FABP and the coding region of the human growth hormone gene to map the cis-acting elements which regulate the developmental stage- and cell lineage-specific and the regional patterns of expression (299,300). Analyses of intestinal isografts have shown that the differentiation-dependent of intestinal FABP synthesis and the horizontal variations in enterocyte intestinal FABP levels do not relate to luminal signals (301).

Studies in transgenic mice indicated that nucleotides -1178 to +28 of rat intestinal FABP gene contain all information to direct cellular and regional patterns of expression of reporters hGH (299) and SV-40 T antigen (73) which are comparable to intestinal FABP in late fetal life. Nucleotides -103 to +28 of the rat intestinal FABP gene are sufficient to direct lineage-

appropriate expression and to establish a duodenal-colonic distribution of reporter hGH mRNA accumulation in enterocytes which mimics that of mouse intestinal FABP mRNA (300). They lack however elements, that sustain expression beyond the suckling/weaning period. Elements between nucleotides -1077 and -277 appear to function as positive transcriptional elements for expression during postnatal life. They also support expression in ileum and colon but a domain located between -277 and -185 suppresses this activation of ileal and colonic expression (300). Removal of this domain yielding a -184 to +28 construct caused inappropriate expression of the transgene in stomach and in nonproliferating and proliferating epithelial cells of the upper half of the crypts (300).

Transfection of Caco-2 cells with fusion genes of elements of intestinal FABP promoter and hGH gene caused expression of hGH in these cells, which usually do not produce intestinal FABP (298). The highest expression was obtained with the shortest construct, of nucleotides -103 to +28, which did not give FABP expression in colon.

Liver FABP gene

The transcription unit of the liver FABP gene spans a region of 3.8 kb encoding 4 exons of 23, 57, 31 and 16 amino acids, respectively separated by three exons of 1.5, 1.2 and 0.6 kb (302)(Fig. 4). Using the rat liver FABP cDNA as a probe the mouse gene was detected in Southern hybridization experiments. This gene is localized on mouse chromosome 6 within 3 cM of the lymphocyte antigen-2 (Ly-2) (247,303) (Table 8). The human gene is localized on chromosome 2 in the p12-q11 region (247, 304).

Fig. 7 shows some of the regulatory elements of the rat liver FABP gene as revealed by studies of Gordon and his coworkers (302,305). Survey of the first 600 nucleotides of the 5'-nontranscribed domain shows besides one TATA-box, a purine-rich region located at nucleotides -53 to -47 which contain the core recognition sequence for members of the Ets superfamily. A peroxisome proliferator response element (PPRE) is present at nucleotides -76 to -66. It differs by one nucleotide from an hepatic nuclear factor 2 (HNF2) binding site in other promoters. The PPRE element will be discussed later. Two additional HNF1 binding sites can be observed. A putative "GATA" binding site is present at nucleotides -245 to -240. C/EBP α protects three regions of the promoter

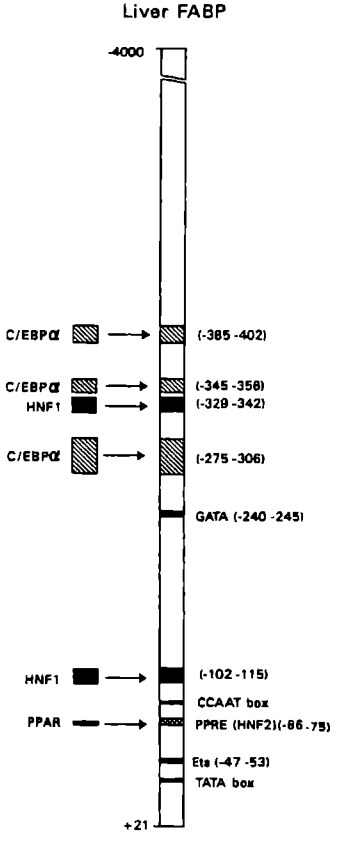

Fig. 7 Cis-acting elements and trans-acting factors in the 5' flanking region of the liver FABP gene.

of the rat liver FABP from DNase I digestion, but these regions do not contain any of the highly diverse C/EBP α binding sites characterized previously.

Expression of the liver FABP gene is confined in the mouse to hepatocytes of the liver (239) and to enterocytes and a subset of enteroendocrine cells of the small intestine (65,73,299, 305). In rat and human liver the liver FABP type is also only present in hepatocytes (88,306-308) but in the intestine of these species it is besides in small intestine also present in the epithelial cells of the cecum and colon (305,309). The liver FABP type was also found in cells of the rat stomach, depending on developmental stage (77,310). In the rat and human kidney also expression of liver FABP type is present with a marked difference in cellular distribution (58, 59). The liver FABP type and its mRNA show a declining gradient of expression from periportal to pericentral hepatocytes in mouse (239), rat (88,237,238,306,311,312) and human (307). All species show besides zonal variations in liver, also a crypt to villus tip gradient in their intestine (72-75,239,301,309). The liver FABP-positive enteroendocrine cells of the mouse intestine appear to represent a small subpopulation of serotogenic cells (313).

The temporal and regional patterns of liver FABP type expression are similar in the rat intestine to that of the intestinal FABP type, although the prenatal mosaic pattern of cellular expression was different (76). Immunoreactive liver FABP and its mRNA are first detectable at fetal day 17 in mouse liver and small intestine and exhibit variations along the cephalo-caudal axis from late fetal life through adulthood (73,301,314). A vertical gradient in cellular liver FABP is also established with the first emergence of nascent villi (73). The content of liver FABP in rat liver increases from fetal day 16 up to adulthood (70,283,284,286). FABP-immunoreactive hepatocytes and enterocytes are present in human at week 7 and 23 of gestation, respectively (307,309). The FABP mRNA level, low in prenatal rat liver and intestine rises markedly around birth and again between 35 and 70 days (286). Liver FABP mRNA was present in similar concentrations in human liver between the 8th through 25th weeks of gestation (315). Studies of fetal and adult intestinal implants into the subcutaneous tissues of adult nude mouse recipients have shown that exposure to luminal contents is not necessary to obtain region- and differentiation-dependent expression of liver FABP type (301).

Liver FABP type appeared to be the only FABP type present in a human colon carcinoma (CaCo-2) cell line (316,317) and to increase with differentiation (91,316). These cells only contain liver FABP mRNA and no intestinal FABP mRNA (247,318).

Analysis of the 5' flanking region of the liver FABP gene was performed in a similar approach as for the intestinal FABP gene by use of fusion genes of sequential deletions of this region and a reporter gene in mice (239,305). A fusion product of nucleotides -596 to +21 with the coding region of hGH or human decay-accelerating factor/HLA-B44 appeared to be sufficient to direct expression of the reporter to hepatocytes and to enterocytes of intestinal epithelium (239,319). However, anomalous expression was observed in cecum, colon, renal proximal tubular epithelial cells and intestinal crypts and in many enteroendocrine cells of young adult mice (239,305,313,319). In fetal mice, nucleotides -596 to +21 are however sufficient to reproduce an appropriate temporal, cellular and regional pattern of reporter

expression (316) like nucleotides -4000 to +21 (73). This larger construct did not give hGH expression in adult mice in colon, kidney and some enteroendocrine cells (239,305,313). It directed a proper horizontal gradient of hGH expression in the intestine, but inappropriate expression occurred in the crypt epithelium, where liver FABP normally is not present.

In liver of adult mice, nucleotides -4000 to +21 produced a pattern of cell-specific and intralobular expression (hepatocytes with a gradient from portal triads to central veins)(299), but the temporal pattern of induction of hGH expression in fetal liver appears to be different from that of liver FABP (73). The nucleotides -132 to +21 of the rat liver FABP gene are sufficient to confine expression to hepatocytes (305). In addition, nucleotides -246 to +21 direct appropriate regional patterns of reporter expression in the hepatic acinus and cell-cycle-dependent variations like for liver FABP were observed (305).

Nucleotides -103 to +21 are already sufficient to direct a pattern of reporter hGH mRNA in villus-associated enterocytes along the duodenal-to-ileal axis of the gut of adult mice that sembles the pattern of the endogenous liver FABP gene (305). This transgene is also expressed in the stomach mucous cells and the colonocytes of the surface epithelium, like the endogenous donor liver FABP gene of the rat, but not the mouse liver FABP gene. It is precociously activated in the intestinal crypt cells and is also expressed in proximal tubular epithelial cells.

Analysis of mice containing different transgenes with sequential deletions of the 5' non-transcribed domain of the liver FABP gene indicated that cis-acting suppressors and activators of cecal and colonic expression are located between nucleotides -4000 and -1600 and between nucleotides -597 and -351, respectively (305). Sequences located outside of -4000 to +21 of the liver FABP gene are necessary to suppress the precocious activation of hGH production along the crypt-to-villus and crypt-to surface epithelial cuff axis (239,305).

The nucleotides -186 to -133 cause a relative suppression of renal expression. The transgene with the -132 to +21 construct showed a marked increase of hGH mRNA in kidney relative to in proximal jejunum, if it was compared with the -186 to +21 construct (305). The region of -4000 to -597 contains cis-acting suppressor elements of renal expression (299) but also more distal sequences appear to be necessary for complete suppression in the mouse (305).

At least seven functionally distinguishable cis-acting elements, located between nucleotides -4000 and +21 influence the number and type of enteroendocrine cells that support liver FABP gene expression (305). Distinct cis-acting sequences appear to be responsible for regulating transcription of this gene in enterocytes and enteroendocrine cells (313).

The content of the liver FABP type appears to be more affected by changes of physiological and pharmacological conditions than the other FABP types (90,283,284,320). Sex and clofibrate affect the content of liver FABP but not of intestinal FABP in intestine (320). Peroxisomal proliferators increase the liver FABP content in liver and intestine of rodents (70,283,320,321) but have no effect on heart FABP content in rat heart and skeletal muscle (283). They rise the liver FABP mRNA level (320,322) by an enhanced transcription rate of the liver FABP gene (322). High-fat diets increase the FABP content of rat liver (46,283,323-325),

adipose tissue (283) and intestine (326). Only one study was reported on liver FABP mRNA during high-fat diet (327). In rat intestine no change of this mRNA was observed and a slight increase of intestinal FABP mRNA.

The upregulation of liver FABP content and FABP mRNA by peroxisomal proliferators and high fat diet in liver and intestine is accompanied by a marked increase of total and especially peroxisomal fatty acid oxidation (283,320,321,328). A significant correlation was observed between the FABP content and peroxisomal oxidation capacity of liver of rats fed with various peroxisomal proliferators (321).

Time courses of induction of liver FABP and peroxisomal oxidation were only studied in cultured hepatocytes. The first studies showed that the induction of FABP and peroxisomal oxidation are temporally consecutive processes (156). Others reported a coordinate increase of FABP, acyl-CoA binding protein and peroxisomal ß-oxidation after three days of treatment with clofibric acid (329). The same was observed for liver FABP, its mRNA, peroxisomal ß-oxidation and cytochrome P450 IVA1-activity (330). Induction of the latter activity measured as lauric acid ω-hydroxylase activity preceded induction of both FABP and peroxisomal oxidation. Hexadecanedioic acid that is poorly metabolized in hepatocytes and non-oxidizable dicarboxylic analogs induce peroxisomal ß-oxidation and liver FABP but not lauric acid ω-hydroxylase, via a pretranslational mechanism, that was not inhibited by inactivators of cytochrome P-450 IVA1 (330,331). These results indicate that the induction is mediated, to a significant extent, by poorly metabolized dicarboxylic acids formed via the cytochrome P450-IVA pathway (330).

The cellular response to peroxisome proliferators in liver and to a less extent in kidney is characterized by an increase in the transcription of several genes that are involved in peroxisomal fatty acid oxidation (332, 333) and of the genes of microsomal cytochrome P450 IVA1 enzymes (330,334,335) and liver FABP (320,322,331). Peroxisomal proliferators as fibrates reduced the expression of mRNAs for the apolipoproteins A-I, A-II and A-IV in rat liver, not in intestine and affected in different directions the expression of enzymes involved in lipoprotein metabolism (336). Non-ß-oxidazable fatty acids (337) and high-fat diets, especially those containing certain poorly metabolizable fatty acids (338-341) also induce the peroxisomal oxidation enzymes, although the mechanism is not known. The common feature of all diverse reagents inducing peroxisomal proliferation is the presence of an acid function or easy conversion to a compound with an acid function (342,343) The mechanisms by which all these chemically distinct compounds work is unclear.

Recently good evidence is arising, that peroxisomal-proliferator-activated receptors (PPARs) mediate the biological action of these compounds. After the first description of murine PPARα (344) several other forms of PPARs have been demonstrated, one in rat, α (345), three in Xenopus, α, ß and γ (346,347) and one human form, Nuc1 (348). The PPARs show considerable sequence similarity, especially in the DNA-binding C domain and the ligand-binding E/F domain. Based on the amino acid sequence of the P-box, which determines DNA recognition specificity (349) they belong to the nuclear hormone receptor subgroup, which comprises receptors for all-trans-retinoic acid (RAR), 9-cis-retinoic acid (retinoic X receptor;

RXR), thyroid hormone, vitamin D_3 and several orphan receptors. All of these receptors recognize the canonical DNA response element AGGTCA (346,349), that is complementary to the peroxisome proliferator reponse element (PPRE) TGACCT (350-353). Such PPRE sequences have been identified in the 5' flanking regions of the genes of rat acyl-CoA oxidase (347,352,
354), of peroxisomal bifunctional enzyme enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (355) and of liver FABP (305,356).

The similarity of the PPAR with other receptors of the nuclear hormone receptor family enabled the investigation of PPAR activators (but not necessary their ligands). By the use of chimeric receptors containing the putative ligand-binding domain of PPAR and the N-terminal and DNA-binding domain of a steroid hormone (oestrogen or glucocorticoid) receptor, specific activators of PPAR could be detected as saturated, monounsaturated and polyunsaturated fatty acids (345,347,353,357) and various peroxisome proliferators, among which Wy-16,343 was most active (347,353,358). Polyunsaturated fatty acids and a synthetic archidonic acid analogue, 5,8,11,14-eicosatetraynoic acid were very effective activators (357). The natural PPAR ligands are still not known. The strong activation of PPAR by a metabolically stabilized fatty acid as tetradecylthioacetic acid, indicates that the weak activity of fatty acids is due to their rapid metabolism within the cell (353). Hexadecanedioic acid was equally potent as the most active fatty acids (353,359).

Diverse PPARs bind to the PPREs of the genes of rat hydratase/dehydrogenase and acyl-CoA oxidase but differentially induce expression (360). Heterodimerization with the RXR causes cooperative and strongly enhanced binding of PPAR and more stimulation of the acyl-CoA oxidase gene promoter (353,357,360). PPAR α binding on PPRE and cooperativity with RXR and other cellular factors appear to be obligatory, but not necessarily sufficient for peroxisome proliferator-dependent transcription induction (360). Distinct PPREs can selectively mediate induction by specific PPARs. Since the presence of 9-cis retinoic acid enhances synergistically with RXR the activity of PPAR, RXR contributes to stimulation of transcription, not only DNA binding (353). The induction of peroxisomal β-oxidation genes by retinoic acid in cultured hepatocytes (361) suggests also a coupling between PPARs and RARs. Peroxisome proliferators and thyroid hormone appear to operate by way of distinct nuclear receptors (362).

In relation with FABP it is important that many peroxisome proliferators (155-157,363), all fatty acids and also hexadecanedioic acid (363) are competing ligands for liver FABP. PPAR activators (ligands) may originate from or are controlled by FABP. In this way FABP may indirectly regulate gene expression. This may involve expression of its own gene.

Since FABP appears also to bind steroid hormones (363,364) an indirect influence on the steroid hormone receptor appears also possible. Oestrogens increase the concentration of FABP in rat liver (365) and of liver FABP mRNA in cultured human hepatoma (HepG2) cells (366). Other indications for involvement of FABP in growth are the increase of liver FABP (237,238) and of the activity of the liver FABP promoter (239) in hepatocytes during mitosis. The growth of hepatoma cells transfected with the coding sequence of liver FABP could be stimulated by

linoleic acid but conversion of this acid to oxygenated metabolites was apparently necessary (240). Growth-stimulatory and -inhibitory eicosanoids bind well to liver FABP, some with higher affinity than fatty acids (156-160,363). Liver FABP was identified as the target protein of the metabolites of the liver carcinogens 2-acetylaminofluorene (238,241) and 4-dimethyl-amino-3-methylazobenzene (364). In mouse, liver FABP is identical with a selenium-binding protein (150). Selenium compounds block cell multiplication and inhibit carcinogenesis. All these findings indicate that liver FABP may be involved in growth and differentiation of hepatocytes.

CRBP I and II genes

The human CRBP I gene harbors 4 exons encoding 24, 59, 33 and 16 amino acids, respectively separated by 0.4, 18.3 and 0.7 kb of intron sequences (90) (Fig. 4). The gene was identified in bovine, chicken and frog (90). The gene is located in the segment p11-qter of human chromosome 3 and on mouse chromosome 9 (90,297) (Table 8).

CRBP I mRNA is first expressed in mouse intestinal and liver RNA at fetal day 16 (367). The following days of development and immediately after birth CRBP I mRNA is also detectable in lung, kidney, testes, seminal vesicle, colon, adrenal, spleen, brain and heart RNA (367,368). The level of expression is highest in lung. During postnatal life CRBP I mRNA levels in the various tissues do not show a coordinate expression (367).

The cis-acting elements and trans-acting factors of the 5' upstream region of the CRBP I gene are depicted in Fig. 8. In the 5' flanking region of the CRBP I gene a high number of CpG dinucleotides is present which is the most common site for methylation in mammalian cells (90). The other members of the FABP family do not contain this high content of CpGs in the 5' flanking region of their gene. The TATA box is absent but 6 to 8 copies of the CCG-CCC sequence, the core sequence for the SP-1 transcription factor binding site, are present. Furthermore a 9 bp sequence element which is similar with the SV40 enhancer was found. Smith et al (369) found additional Krox-20 or Krox-24 and two NF-1 binding sites. In the more upstream region a retinoic acid response element (RARE) was found which sequence closely resembled that of the RARE in the RAR ß$_2$ promoter. CRBP

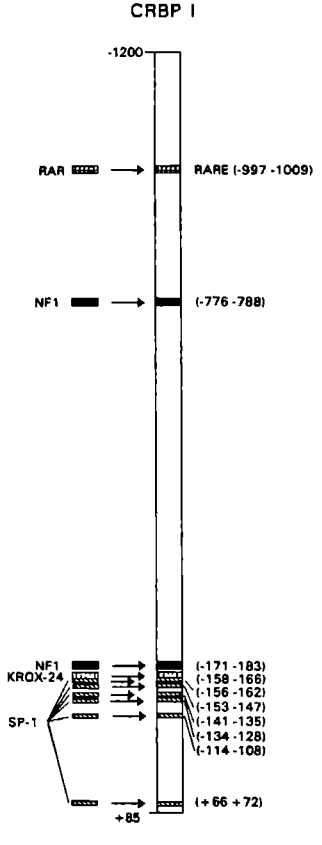

Fig. 8 Cis-acting elements and trans-acting factors in the 5' flanking region of the CRBP I gene.

I expression seems to be activated by RAR, since three RAR isoforms α, ß and γ are able to bind to the RARE in the 5' flanking region of the CRBP I gene (369,370).

The gene for CRBP II was isolated from rat (297). The four exons encoding 24, 59, 34 and 16 amino acids, respectively are interspersed by 3 introns of 5.3, 9.2 and 1.4 kb in length (297) (Fig.4). The gene was identified in human and mouse DNA, but was not detected in chicken and fish DNA (297). The mouse gene is located on chromosome 9 and the human homolog on chromosome 3 (297) (Table 8). In both species the CRBP II gene is closely linked to the CRBP I gene.

In contrast to CRBP I mRNA, expression of CRBP II mRNA is less widely spread. In fetal mouse high mRNA levels were found in intestine and only trace amounts in liver and lung (367). CRBP II transcript expression is first detectable at fetal day 19 simultaneously with the appearance of a proliferative intestine absorptive columnar epithelium (367,371). The expression in intestine declines dramatically after birth, remains low during the weaning period and rises again during adulthood (367,371,372). Expression in liver disappears completely after birth (367,371).

The transcription-regulatory elements, the CAAT- and TATA-box, are both present in the CRBP II gene. As mentioned for intestinal FABP, the CRBP II gene also contains a 14-bp element in the 5' flanking region (297). This element binds the transcription factors HNF4 and ARP-1, which might direct expression of the gene in the intestine (298). The expression of the CRBP II gene is dramatically upregulated by 9-cis retinoic acid by RXR (but not by RAR) via a retinoid X reponse element (RXRE) in the gene promoter region (373). Between nucleotides -639 to -605 five nearly perfect tandem repeats are present which form the RXRE (373).

CRABP I and II genes

The mouse CRABP I gene spans 10.5 kb containing 4 exons encoding 24, 59, 38 and 16 amino acids, respectively which are separated by 3 introns 0.5, 3.5 and 5.5 kb in length (374) (Fig. 4). The gene was detected by Southern hybridization in bovine, human, chicken, frog and fish (368,375,376). The human CRABP I gene is localized on chromosome 3 like CRBP I and II. Since no linkage studies were done it is not known if the CRABP I gene forms a cluster with the CRBP genes (375).

CRABP I mRNA is expressed in testis, seminal vesicle, brain, kidney and skin of adult rat (377). The expression pattern is similar in neonatal rats but in general the transcription level is higher. In mouse, CRABP mRNA was only detected in seminal vesicle RNA (368).

Analysis of the 5' flanking region of the CRABP I gene revealed that no TATA box was present. A high GC content and multiple copies of the putative SP1 transcription factor binding sequence were found identical with CRBP I and housekeeping genes (374).

The CRABP II gene was isolated for mouse and man with a length of 4.6 and 6 kb, respectively. The length of the three introns are 3-4, 0.2 and 0.5 kb, respectively (250,251). The gene has been mapped to mouse chromosome 2, approximately 4.2 cM distal to the muscle nicotinic acetylcholine receptor α-chain gene and 4.5 cM proximal to the catalase-1 gene (250).

For human it could therefore be located on chromosome 2 or 11 (Table 8).

CRABP II mRNA is only expressed in skin of neonatal rats. Its level is approximately 5% of CRABP I expression in the same tissue. In adult rats no transcripts were detected in skin and only trace amounts of mRNA in adrenal (377).

The cis-acting elements and trans-acting factors controling CRABP II gene expression are shown in Fig. 9. The 5' flanking region of the mouse CRABP II gene contains a TATA-box, a GC-rich region with two overlapping SP-1 recognition motifs, an AP-1 consensus and an AP-2 consensus (250). In addition a direct repeat spaced by 1 bp was found with similarity to the RARE in the RAR β_2 gene (251). Two potential AP-2 binding sites, one SP-1 site and two Krox-24 binding sites are present in the upstream region of the human gene (251). Durand et al. (378) found more upstream a second RARE.

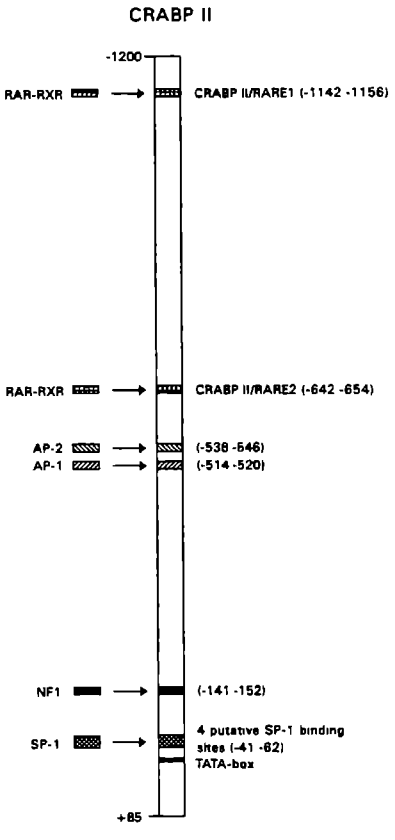

Fig. 9 Cis-acting elements and trans-acting factors in the 5' flanking region of the CRABP II gene.

Since we focus on FABPs in this review we will only briefly discuss the interaction of the CRABPs with nuclear retinoic acid receptors. For an extensive discussion see refs 39 and 379-382. These receptors belong to the steroid/thyroid hormone nuclear receptor superfamily and can be divided in receptors with high affinity for all-trans retinoic acid, the RAR and for 9-cis retinoic acid, the RXR. Both have three subtypes α, β and γ, which have several isoforms generated by alternative splicing (382). Upon activation the RAR and RXR form dimers and recognize specific cis-acting elements RARE and RXRE in the 5' upstream region of genes which are transcriptionally activated. RAR and RXR form homodimers but also heterodimers, both among themselves and with other members of the hormone nuclear receptor super family (353,357,360,373,379,383). Heterodimer RAR and RXR (RAR-RXR) bind more efficiently than homodimers to the RAREs (378). Dollé and coworkers (384) showed a differential and tissue-specific expression of the three RAR genes and the genes of CRBP I and CRABP I. They suggested that this spatio-temporally regulated expression during development may account for the diversity of retinoic acid effects. Due to this differential expression the activation of gene transcription of developmental genes also varies in the cells. The role of CRBP and CRABP in embryonal development may be controlling the free retinoid concentration and thereby the activity of the RAR and RXR (384). CRBP may regulate the concentration of free retinol and its conversion to retinoic acid. CRABPs may sequester

retinoic acid which leads to a difference in availability of retinoic acid for RAR and RXR in the various cells where CRABPs are expressed (380,385). Retinoic acid induces the expression of the genes of CRABP I and II, RAR ß, laminin B1 and collagen type IV in F9 teratocarcinoma cells (385). Stably transfected cell lines expressing elevated levels of CRABP I showed a 80-90% reduction in the retinoic acid induced expression of RAR ß, laminin B1 and collagen type IV.

The CRBP and CRABP levels themselves are also regulated by retinoic acid. Response elements for RAR and RXR are present in the 5' upstream region of the CRBP I (369) and the CRBP II gene (373), respectively. The CRABP II gene promoter has a response element which is recognized by a heterodimer of RAR and RXR (378). The promoter activation of the CRABP II gene is more efficiently increased by 9-cis retinoic acid than by all-trans retinoic acid (378). The CRABP II gene expression in F9 teratocarcinoma cells is not directly regulated by retinoic acid as was shown by nuclear run on experiments and the posttranscriptional inhibition of induction by cycloheximide (250,251). It was suggested that a newly synthesized regulatory protein is involved in induction of CRABP II expression (251).

Conclusions

The abundance of FABP and FABP mRNA in the cytosol of various cells of vertebrates and invertebrates indicates the importance of these molecules. Seven FABP types have been identified on the protein and/or RNA level. Some are present in only one cell type or tissue, other, especially the heart type, have a more broad tissue distribution. The existence of different types can be explained by tissue-specific or functional adaptations. The tertiary structure of the investigated FABP types shows a similar ß-clam, but the fatty acid-binding site appears to differ. The structure of the liver FABP type is not known, but appears rather unique, since this molecule has a different primary structure and also shows different properties with biophysical techniques and various ligands. The liver FABP type binds, in contrast to the heart type, besides long-chain fatty acids also many other hydrophobic ligands, including haem, some prostaglandins, leukotrienes, bile acids and peroxisomal proliferators.

The function of FABPs in fatty acid uptake and intracellular transport and in fatty acid metabolism needs more conclusive evidence. Recent observations indicate also a direct or indirect role in complex processes as cellular growth, differentiation and signal transduction as well as a protective role with respect to membranes and enzyme systems. A primary role of FABPs in pathology has not been reported.

The increasing knowledge on the structure of FABPs, their mRNAs and genes and the use of sophisticated molecular-biological, cell-biological and biophysical techniques will allow in the near future a better understanding of the biologic significance of the different FABP types.

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CHAPTER 3

TWO TYPES OF FATTY ACID-BINDING PROTEIN IN HUMAN KIDNEY

Isolation, characterization and localization

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SUMMARY

Two types of fatty acid-binding protein (FABP) were isolated from human kidney by gel filtration and ion-exchange chromatography. Characterization based on molecular mass, isoelectric point, fluorescence with dansylamino-undecanoic acid and immunological cross-reactivity showed that one, type B, was fairly similar to human heart FABP. The other, type A, showed, like human liver FABP, a high fluorescence enhancement and a wavelength shift with dansylamino-undecanoic acid as well as the binding of a variety of ligands. Antibodies raised against FABP type A and against liver FABP markedly cross-reacted in e.l.i.s.a., in Western blotting and in indirect immuno-peroxidase staining on kidney and liver sections. Differences in amino acid composition and isoelectric points, however indicate that type A might be a kidney specific type. The FABP type A is more abundant in kidney than the B type and is predominantly localized in the cortex, especially in the cells of the proximal tubules. The FABP type B is mainly present in the cells of the distal tubules.

In conclusion, this study shows the presence of two types of FABP in the kidney. One type seems to be related to heart FABP, while the other type resembles, but seems not identical to, liver FABP. Both types have a characteristic cellular distribution along the nephron.

INTRODUCTION

In many tissues a low-molecular mass fatty acid-binding protein (FABP) is present. A precise function of FABP has not yet been shown, but it is generally assumed that it is involved in fatty acid transport and metabolism [1-5]. In man three FABP types have been identified thus far: heart [6-8] liver [9,10] and intestinal FABP [11]. In rodents five distinct FABP types have been isolated and characterized. Immunochemical and molecular-biological studies show that heart FABP and/or its mRNA are present in skeletal muscle, kidney and many other tissues, but not in liver and intestine [2,4,5,12-14]. The liver type FABP is located in hepatocytes and shows in the intestine a gradient from duodenum to colon and from crypt to villus tip [15-17]. Intestinal FABP is similarly distributed within the intestine, but is not present in liver [18,19]. A specific lipid-binding protein is present in adipocytes [20]. Fujii et al. [21] isolated an FABP from rat kidney, which differed from rat liver FABP. Recently Lam et al. [22] showed the presence of a second renal-specific FABP in rat kidney and bladder, besides the heart type.

In experimental hypertension the content of the renal FABP type is decreased in rat kidney in contrast with that of the heart type [22]. Other investigations showed that in this condition the content of heart FABP is also decreased in rat aorta [23]. In spontaneously hypertensive rats, however, the FABP content of kidney medulla is higher than in normal rats [24].

FABPs may play an important role in fatty acid metabolism of kidney, since fatty acids are a major source of energy in this organ [25-28]. Considerable differences in the FABP content were found between cortex and medulla of rat kidney, but both a higher content of cortex [22] and of medulla [24] were reported. Studies of mammalian nephron segments have shown a large diversity of transport functions [29] and a specific distribution of enzymes of

metabolic pathways [30]. These data suggest that the content and nature of the FABP type may differ in the various cell types of the nephron. Therefore we decided in our series of studies on FABPs from human tissues [6,10,31] to investigate the presence of FABP in the human kidney. In the present study we describe the isolation, characterization and cellular distribution of two FABP types of human kidney.

MATERIALS AND METHODS

Materials

Sephacryl S-200, CM-cellulose, DEAE-Sepharose CL-6B Fast Flow and Sephadex G-50 (fine grade) were obtained from Pharmacia Biotechnologies (Int.), Uppsala, Sweden; Lipidex 1000 from United Technologies Packard, Downers Grove, IL. USA; [1-^{14}C]oleic and [1-^{14}C]palmitic acids from Amersham, Little Chalfont, Bucks, U.K.; 11-dansylamino-undecanoic acid from Molecular Probes, Junction City, OR, USA; polystyrene 96-well flat-bottom microtitre elisa plates from Greiner, Nurtingen, Germany; horseradish peroxidase-conjugated swine anti-rabbit IgG from Dakopatts A/S, Glostrup, Denmark; goat anti-(human Tamm-Horsefall glycoprotein) from Cappel, West Chester, PA, USA; Diaflo YM-2 and YM-5 membranes from Amicon Corporation, Lexington, KY, USA; nitrocellulose sheets (BA-85, pore size 0.45 μM) and electroelution membranes (BT1, BT2) from Schleicher & Schüll, Dassel, Germany; Servalyt ultrathin 5% polyacrylamide Precoates from Serva GMBH & Co, Heidelberg, Germany. Human kidneys were obtained after nephrectomy or at obduction within 15 h after death. Rat kidneys were obtained from 10 week old male Wistar rats.

Methods

Purification of fatty acid-binding proteins

Renal cortex was separated from medulla using the junction of cortex and outer medulla as anatomical marker. Cortex (150-175 g) originating from 2 kidneys was homogenized (40%; w/v) in ice-cold 10 mM-potassium-phosphate/154 mM-potassiumCl (pH 7.4) with a polytron homogenizer. The homogenate was centrifuged for 10 min at 600 g and subsequently for 90 min at 105,000 g. The supernatant (cytosol) was fivefold concentrated by ultrafiltration on a Diaflo YM-5 membrane. The concentrated cytosol was applied, in four portions of 9 ml, to a Sephacryl S-200 gel filtration column (2.6 x 110 cm), equilibrated in 30 mM-Tris/HCl (pH 8.0)/1 mM-dithiothreitol/1 mM-EDTA and eluted with the same buffer. The fractions containing fatty acid-binding activity were combined, dialysed overnight against 5 mM- Tris/HCl (pH 8.4) and applied to a DEAE-Sepharose Fast Flow column (2.5 x 10 cm), equilibrated in the same buffer. The column was eluted stepwise with 0.6 l of 15 mM, 30 mM- and 50 mM-Tris/HCl (pH 8.0). The fractions containing fatty acid-binding activity were combined, concentrated on a Diaflo YM-2 membrane and applied to a CM-cellulose column (4.8 x 4 cm), equilibrated in 20 mM-potassium-phosphate/1 mM-dithiothreitol (pH 5.4). The column was

eluted with 0.3 l of the same buffer. The fatty acid-binding fractions were combined, concentrated on a Diaflo YM-2 membrane and applied to a Sephadex G-50 (fine) column (1.5 x 70 cm), which was equilibrated and eluted with 10 mM-potassium-phosphate/1 mM-dithiothreitol (pH 7.4). The purified FABPs were stored at -20°C.

FABPs from human and rat liver [10], heart [11] and human skeletal muscle [31] were isolated as described. Heart and muscle FABPs were additionally purified by CM-cellulose chromatography.

Electroelution

FABP preparations obtained from the Sephadex G-50 column, were fractionated by SDS/PAGE in 16 % gel at pH 8.8. After Coomassie Blue staining of the molecular-mass markers and a small strip of the protein sample, the major 15 kDa band was cut from gel. The gel slices were applied to the Biotrap electrophoresis chamber (Schleicher & Schuell, Dassel, FRG) filled with 0.05 M-Tris/25 mM-glycine/0.1 % SDS buffer (pH 6.8) [32]. Electroelution was executed for 2 h at a constant voltage of 200 V. The protein trapped between the BT1 and BT2 membrane was used.

Immunocytochemistry

Kidney slices were fixed in 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4) and embedded in paraffin. All further procedures were done at room temperature. Sections were deparaffinized and treated for 30 min. with 1% (v/v) hydrogen peroxide in methanol to remove intrinsic peroxidase activity. After rehydration, sections were incubated with 20% (v/v) normal swine serum for 15 min followed by rabbit antiserum raised against one type of FABP (diluted 1:250 or 1:500) for 90 min. The sites of immunoreaction were revealed with swine anti-rabbit IgG-peroxidase using diaminobenzidine as a substrate. Sections were counterstained with Harris's haematoxylin and embedded in glycerol/gelatin mounting medium. To localize distal tubules, an antiserum against Tamm-Horsefall glycoprotein was used [33,34]. Pre-immune sera and antisera raised against non-related antigens were applied as controls.

Other procedures

Fatty acid-binding activity of column fractions and isolated FABP preparations were determined as described by Peeters et al. [31]. Amino acid analysis, fluorescence measurements of 11-dansylamino-undecanoic acid binding to FABP and its displacement by various ligands were performed as previously described [6, 10]. Tryptophan determination in 6 M-guanidinium HCl (pH 6.5) and 5,5-dithiobis-(2-nitrobenzoic acid) analysis for determination of free cysteine were performed as described by Edelhoch [35] and Wilton [36], respectively.

Polyacrylamide slab gel electrophoresis was performed in 0.1% SDS at pH 8.8 in 16% acrylamide gels. Reductive gels were run after addition of 10 mM-2-mercaptoethanol in the upper reservoir buffer [37]. Gels were stained with Coomassie Brilliant Blue or silver [38]. Isoelectric focusing was performed on native 5% ultrathin polyacrylamide gels. Protein samples

(5-10 μg) were subjected to focusing at 1000 V in a Desaga Mediphor electrophoresis apparatus. Gels were fixed in 0.82 M-trichloroacetic acid for 15 min and stained with Coomassie Brilliant Blue R250 in solution (methanol/acetic acid/water; 5:1:4 by vol.) for 15 min. Gels were de-stained in the same solution.

Preparation and characterization of the rabbit antisera against the FABPs of kidney were performed as described for the FABPs from heart [6], liver [10] or muscle [31]. ELISA and immunoblotting were as previously described for rat FABPs [39].

RESULTS

Purification of two FABP types

The combined fractions of low-molecular-mass proteins with fatty acid-binding activity obtained after Sephacryl S-200 gel filtration of cortex cytosol were separated on DEAE-Sepharose. After stepwise elution with 15, 30 and 50 mM-Tris/HCl (pH 8.0), respectively two peaks with fatty acid-binding activity were identified, peaks A and B (Fig. 1). Both preparations were applied to a CM-cellulose column to remove contamination with haemoglobin subunits. After CM-cellulose, preparation B was pure by SDS/PAGE, preparation A contained only FABP and a protein of approximately 30 kDa. Gel filtration on Sephadex G-50 did not remove this latter protein.

Therefore the sample was refractionated by preparative SDS/PAGE. After electroelution of FABP (15 kDa band) from the gel and re-electrophoresis, the gel again showed FABP and the 30 kDa protein. These results indicate that the contaminating protein is a

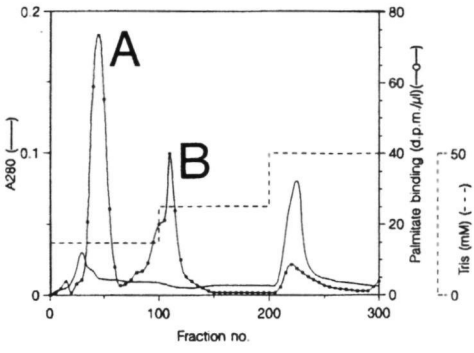


Fig. 1. Purification of FABPs from the low molecular-mass fraction of the renal cortex cytosol by DEAE-Sepharose anion-exchange chromatography.
Bound proteins were eluted stepwise with 15, 30 and 50 mM Tris/HCl (pH 8.0). Protein was monitored at 280 nm (———) and 300 μl samples were assayed for [1-^{14}C]palmitic acid binding (o).

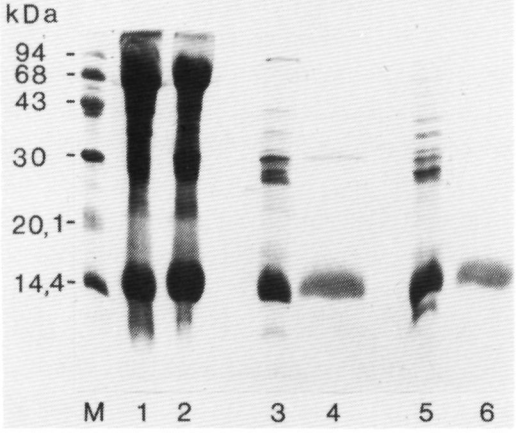


Fig. 2 SDS-PAGE of preparations at different stages of the purification of FABP from human renal cortex.
The gel was stained with Coomassie Brilliant Blue.
Lanes labelled with "M" contain calibration proteins (Mr): phosphorylase b (94000); BSA (67000); ovalbumin (43000); carbonic anhydrase (30000); soybean trypsin inhibitor (20100); and α-lactalbumin (14400). Lane 1; cytosolic protein (200 μg); lane 2; combined FABP-containing fractions after Sephacryl S200 gelfiltration (200 μg); lane 3; peak A after DEAE-Sepharose chromatography (40 μg); lane 4; FABP type A after CM-cellulose chromatography (15 μg); lane 5; peak B after DEAE-Sepharose chromatography (40 μg); lane 6; FABP type B after CM-cellulose (15 μg).

dimer of FABP, as was also observed previously with rat liver FABP [10,39,40].
The results of a typical purification procedure are summarized in Table I and Fig. 2. The yield of FABPs is rather low but is in the same order as for human liver [10] and human heart [6]. The ratio of preparations A and B was always approximately 4:1. Purification from kidney cortex of both female or male individuals showed the same results. Purification of FABP from the cytosol of kidney medulla yielded comparable amounts of the B preparation but only slight amounts of the A preparation.

Characterization of the FABP preparations

The FABP preparations obtained as peak A and B appeared to be no isoforms, but different FABP types (A and B). The molecular mass determined by gel electrophoresis is 14.7 ± 0.2 (mean ± SD, n=6) kDa for type A and 16.1 ± 0.2 kDa (n=6) for type B. Isoelectric focussing revealed an pI of 6.8 for type A and of 5.2 for type B, respectively. These isoelectric points agree with the different elution positions of the proteins from DEAE-Sepharose.

Table 1 Isolation of FABPs from human renal cortex. Protein was determined by the procedure of Lowry et al. [54] or by quantitative amino acid analysis (after CM-cellulose and Sephadex G-50); fatty acid-binding activity was assayed at 1 μM-[1-^{14}C] oleic acid. The supernatant was freed from albumin by affinity chromatography on Sepharose-anti-(human serum albumin) before assay of specific binding activity.

Purification step	Protein (mg)	Specific binding activity (nmol/mg)	Purification (-fold)	Yield %
105000 g supernatant	10298	0.55	1.0	100
Sephacryl S-200	808	2.53	4.6	36
DEAE-Sepharose				
Preparation A	49	5.52	10.0	4.8
Preparation B	12	6.06	11.0	1.3
CM-cellulose				
Preparation A	21	17.5	15.9	3.3
Preparation B	5	27.4	24.9	1.2
Sephadex G-50				
Preparation A	5	16.4	14.7	0.7

The amino acid compositions of both proteins were compared with each other and with our data on human liver and heart FABPs [6,10].The FABP type A differs slightly from liver FABP but is markedly different from heart FABP (Table 2). The FABP type B preparation had a composition, which differed from kidney type A FABP and from liver and heart FABPs. On base of a molecular mass of about 14.5 kDa, FABP type A and liver FABP contain one cysteine residue in contrast with the other FABPs. Determination of tryptophan however, showed that FABP type A contains two tryptophan residues, whereas liver FABP contains none.

Table 2 Amino acid composition of FABPs from kidney (A and B types), liver and heart
Values are given in residues/1000 amino acid residues and are means±S.D. for the number of preparations in parenthese. Data on human heart and liver FABPs were obtained from Paulussen et al. [6] and Peeters et al. [10] respectively. Statistical significance: different from liver FABP: $^{a}P<0.001$, $^{b}P<0.01$; different from heart FABP: $^{c}P<0.001$, $^{d}P<0.01$; different from kidney FABP type B: $^{e}P<0.001$, $^{f}P<0.01$.

Amino acid	Kidney FABP type A (n=8)	Liver FABP (n=5)	Heart FABP (n=3)	Kidney FABP type B (n=4)
Asp	106±5[b,f]	94±5	108±4	96±5
Thr	67±2[a,f]	78±5	127±10	73±2[e]
Ser	61±6[e]	59±7	54±5	39±4[d]
Glu	152±3[e]	158±10	102±5	115±16
Pro	37±3[e]	31±6	18±4	62±2[e]
Gly	100±5[e]	95±3	84±1	153±6[e]
Ala	60±4[e]	51±9	57±3	71±3[d]
Cys	8	ND	ND	ND
Val	71±4[e]	65±11	63±4	59±2
Met	18±2	15±3	17±4	16±2
Ile	65±7[e]	63±11	33±6	44±2
Leu	67±3	69±5	104±3	69±3[e]
Tyr	17±2[e]	16±3	16±1	10±1[e]
Phe	54±3[e]	56±2	46±4	39±2
Trp	16±1[e]	0	9±1	ND
His	54±3[a,e]	15±3	25±3	17±2[d]
Lys	99±3[b,f]	109±8	107±8	92±2
Arg	24±2[e]	24±3	32±2	44±1[e]

Fatty acid-binding studies

The binding affinities (Kd values) for oleic acid of type A and B were 0.2 and 0.3 μM, respectively. The maximal binding capacity of preparations of both proteins obtained in different isolation procedures was 16-30 pmol of oleic acid/μg FABP (0.24-0.45 mol/mol FABP). The cause of the deviation of the binding ratio from 1.0 may be the loss of binding activity by the ion-exchange chromatography as previously observed with FABPs isolated from other tissues [6,10, 11,41].

Measurements of the interaction of FABP type A with 11-dansylamino-undecanoic acid by fluorescence showed a marked shift of both excitation (328-345

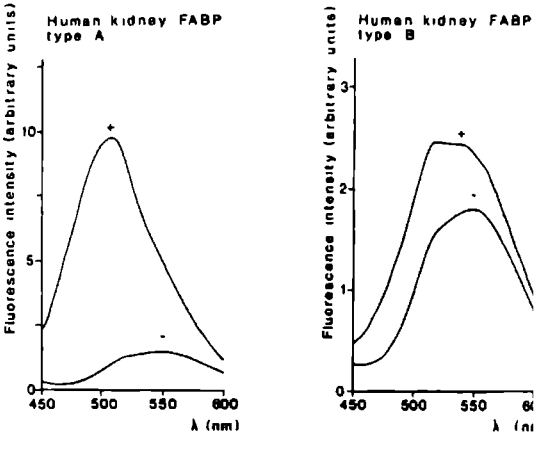


Fig. 4 Fluorescence emission spectra of 1 μM 11-dansylamino-undecanoic acid with (+) or without (-) 2.7 μM kidney FABP type A or B in 10 mM Tris/HCl (pH 8.0). Excitation wavelengths were 330 and 345 nm, respectively in the absence and presence of FABP type A. No shift in excitation wavelength was found for FABP type B.

nm) and emission (545-505 nm) wavelengths and a large enhancement of fluorescence (Fig.4), similar to observations with liver FABPs [10]. Kidney FABP type B did not show the wavelength shifts and only a slight enhancement of fluorescence (Fig.4), comparable with heart FABP [10].

Displacement studies with various types of ligands were performed to investigate the binding specificity of the FABP preparations. Various ligands caused a displacement of the fluorescent probe from renal FABP type A similar to that from human liver FABP (Fig.5). The type B did not show this phenomenon and appeared to be more ligand-specific similar to heart FABP [6].

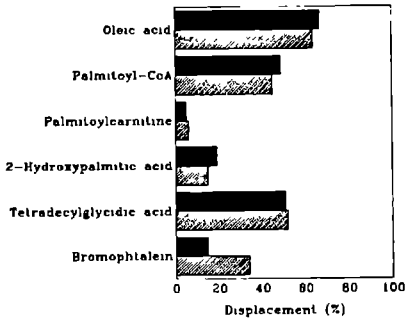


Fig. 5 Displacement of 11-dansylamino-undecanoic acid from human liver FABP and kidney FABP type A by different ligands. Concentrations were 40 μg FABP, 1.0 μM dansylamino-undecanoic acid and 1.0 μM fatty acid (1% ethanol) in 1 ml 10 mM Tris/HCl (pH 8.0). Fluorescence was measured at 345 nm (excitation) and 505 nm (emission). Displacement (decrease of fluorescence) is given as a percentage of the control fluorescence.

Immunochemical studies

The specificity of the antisera against the kidney FABPs was assayed on Western blots of cytosols from kidney cortex. They revealed only two bands at 14.5 kDa and 30 kDa with anti-(FABP type A) antiserum and one band at 15.5 kDa with anti-(FABP type B) antiserum. The former observation was in accordance with the presence of a dimer, as indicated above. The cortex cytosol also reacted with antisera against FABPs from human liver and heart, similar to the anti-(type A) and anti-(type B) antisera respectively. The renal FABP types and their antisera showed slight cross-reactivity. Human liver and heart FABP reacted only with anti-(FABP type A) and anti-(FABP type B) antiserum respectively.

Table 3 Immunological cross-reactivity of FABP and FABP antisera of human kidney, heart and liver. Serum dilutions at which half-maximal binding was obtained in an elisa were taken as measures for the immunological cross- reactivity. Per well, 0.4 μg of FABP was used. Values are means $\pm$ S.D. of at least three experiments and are given as percentage of the binding of the specific antiserum to the specific antigen.

Origin of FABP	Antiserum against FABP from:	Reactivity (%)			
		Kidney type A	Kidney type B	Heart	Liver
Kidney type A		100	2±0	0	85±8
Kidney type B		22±5	100	88±1	13±1
Heart		3±1	91±7	100	8±2
Liver		67±7	2±0	4±2	100

The cross-reactivity of the renal FABP types and heart and liver FABP and their

antisera was also investigated in ELISA (Table 3). The renal FABP types show a low degree of crossreactivity. The FABP type A cross-reacts strongly with anti-(liver FABP) serum, while the FABP type B does so with anti-(heart FABP) serum.

Tissue distribution of both FABP types

We studied the distribution of both FABP types in sections of human kidney with the antisera against FABP type A and B by the immunoperoxidase procedure. Antisera against FABPs from human liver, heart or skeletal muscle were also applied. Sections of the cortex and medulla were used to study different parts of the nephron. Identification of renal tubules was based on their position and relative abundance and on the morphological characteristics of their epithelial cells such as cell shape, position of the nucleus, presence of a brush border, cell striation and appearance of cell boundaries [42,43]. Distal-tubular cells were identified with the antiserum against Tamm-Horsfall glycoprotein, which is specific for distal tubular cells. The results of the immunolocalization studies are shown in Figs. 6-9 and summarized in Table 4. Pre-immune sera did not show significant staining.

Table 4 Immunohistochemical localisation of FABP along the nephron using antisera raised against different FABP preparations. Staining: ++, strong; +, moderate; -, weak or absent. Abbreviations: Glom, glomeruli; PCT, proximal convoluted tubules; PST, proximal straight tubules; IT, intermediate tubules; DST, distal straight tubules; DCT, distal convoluted tubules; CD, collecting ducts.

Antiserum against FABP from:	Staining intensity along the nephron						
	Glom	PCT	PST	IT	DST	DCT	CD
Kidney (type A)	-	++	+	-	-	-	-
Liver	-	++	+	-	-	-	-
Kidney (type B)	-	+	-	-	++*	++	-
Heart	-	+	-	-	++*	++	-
Psoas muscle	-	+	-	-	++*	++	-

* Macula densa was negative

In human kidney, anti-(FABP type A) antiserum immunoreacted strongly with the proximal convoluted tubules (Fig. 6a), moderately with the proximal straight tubules and weakly or not with the other tubules (Fig. 6b). A comparable distribution was found using anti-(human liver FABP) antiserum (Fig. 7). Interestingly, human fetal kidney contains already early FABP, like human liver [44]. We observed immunoreactivity of proximal convoluted tubules of the kidney of a 4 months-old fetus with anti-(FABP type A) antiserum.

In contrast, the distal tubules were found strongly immunoreactive with anti-(FABP type B) antiserum, while the proximal convoluted tubules showed a moderate staining (Fig. 8). The macula densa, a plaque of specialized tubular cells situated within the end portion of the

distal straight tubule was found negative (Fig. 8a, inset). The other tubules stained weakly or not at all. Using anti-(human heart FABP) or anti-(human muscle FABP) antisera, a similar distribution was obtained (Fig. 9). This is also the case when rat kidney sections were incubated with anti-(rat heart FABP) antiserum.

The cytoplasm of renal adipocytes was strongly immunoreactive with the antisera against renal FABP type B, heart and muscle FABP, but not with the other sera (not shown). On human liver sections, the antiserum against kidney FABP type A reacted strongly with hepatocytes as anti-(human liver FABP) antiserum did. Antisera against FABP type B, heart or muscle FABP gave only a weak or no reaction.

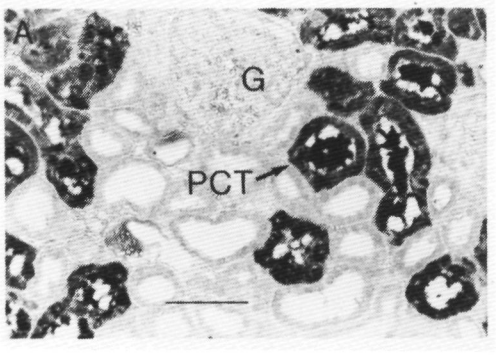


Fig. 6 Immunoperoxidase staining of human renal cortex (a) and medulla (b) using antiserum against type A FABP. Proximal convoluted tubules (PCT) are strongly immunoreactive, while proximal straight tubules (PST) react weakly. Glomeruli (G) and other tubules, e.g. collecting ducts (CD) and distal straight tubules (DST), are negative. The bar represents 80 μm.

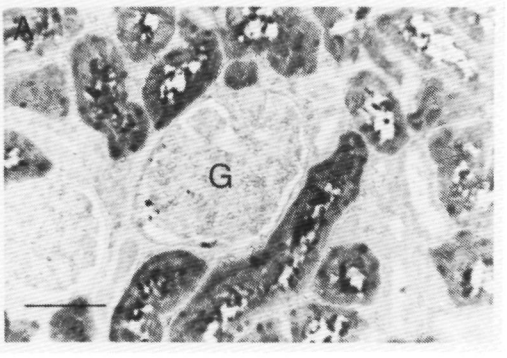
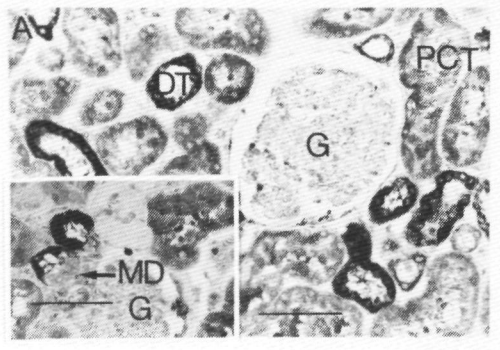
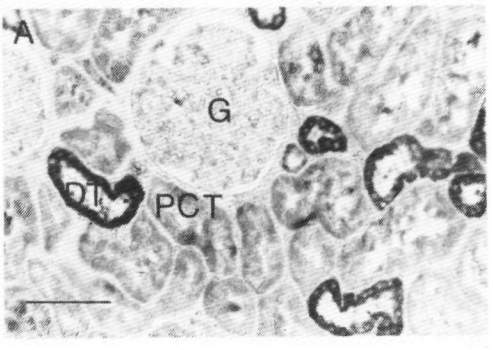
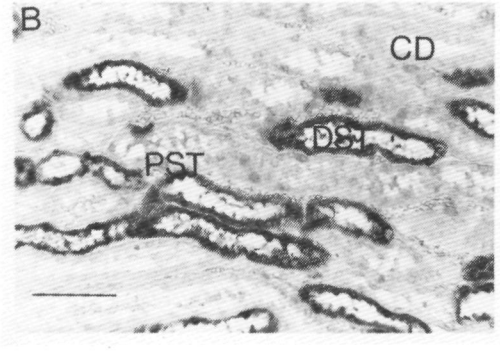


Fig. 7 Immunoperoxidase staining of human renal cortex (a) and medulla (b) using antiserum against human liver FABP. A similar distribution is obtained as for anti-(FABP type A) antiserum (Fig. 6). The bar represents 80 μm.







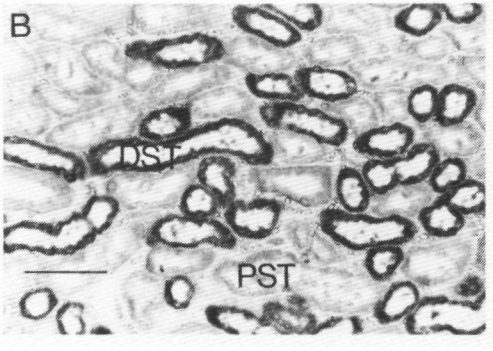


Fig. 8 Immunoperoxidase staining of the human renal cortex (a) and medulla (b) using antiserum against FABP type B. Distal tubules (DT) are strongly positive, while proximal convoluted tubules (PCT) show a moderate staining. Other tubules e.g. proximal straight tubules (PST), collecting ducts (CD) and glomeruli (G), are negative. Inset: the macula densa (MD), a specialized region of the distal straight tubule, does not stain. The bar represents 80 μm.

Fig. 9 Immunoperoxidase staining of human renal cortex (a) and medulla (b) using antiserum against human heart FABP. A similar distribution is obtained as for anti-(FABP type B) antiserum (Fig. 8). The bar represents 80 μm.

DISCUSSION

Two types of FABP, type A and type B, were found in human kidney. FABP type A resembles human liver FABP [10] in many, but not all, respects. It has a molecular mass of 14.5 kDa and shows a 30 kDa dimer band similar to human and rat liver FABPs [10,39,40]. The dimer may be caused by apolar interactions rather than by disulphide bridges since it is still present when SDS/PAGE is performed under strongly reductive conditions [37]. The Kd values for oleic acid are of the same order for type A and liver FABP. Fluorescence with 11-dansylamino undecanoic acid and the displacement of this fatty acid by various ligands are similar to

those of liver FABP [10]. Both, FABP from human liver and kidney (type A) had a lower affinity for palmitoyl-CoA than for fatty acids, similar to rat and bovine liver FABP [10,45,46,47]. Preparations did not contain acyl-CoA binding protein (Mr 10 kDa).
Antisera raised against renal FABP type A and liver FABP showed a high cross-reactivity with both FABPs and are immunoreactive with the same cells within the kidney and liver. Antisera raised against the FABPs from rat kidney, however, did not react with rat liver FABP [21,22]. Rat kidney FABP has been reported to be identical with α_{2U}-globulin, which is only present in male rat kidney [48]. We isolated both FABP types from male as well as female kidney cortex.

The amino acid composition as well as the isoelectric point differ between FABP type A and liver FABP. The former contains two tryptophan residues whereas the latter does not. The isoelectric point of type A is more neutral than that of human liver FABP (pI 5.8) [10]. We therefore conclude that FABP type A might be a new kidney specific FABP type. The human kidney FABP type A contains, similarly to the specific rat kidney FABP type both cysteine and tryptophan in contrast to the liver FABP type.

The other type of renal FABP, type B, seems to be related to human heart FABP, but its amino acid composition differs. The molecular mass (15.5 kDa) and the Kd for oleic acid are similar for human heart FABP [6] and FABP type B. The pI is 5.2, which is comparable with that of the FABPs from human, rat, porcine and bovine heart [6,41,49] and the heart type FABP of rat kidney [22]. Antisera raised against FABP type B and human heart FABP give a high cross-reactivity and are immunoreactive with the same cells within the kidney. Both sera show also reactivity with the cytoplasm of adipocytes. Human adipocytes contain a specific lipid-binding protein, which has a high similarity to heart FABP [20]. The presence of a heart type FABP in human kidney is in agreement with the observations on rat heart FABP and its mRNA in rat kidney [12,14,22,38]. No data, however, are available on the ligand properties of the heart type FABP isolated from rat kidney [22].

On base of the yield of the isolation procedures, cortex and medulla of human kidney appear to differ in the proportion of both FABP types, similar to that observed in rat kidney [22]. Our immunohistochemical data demonstrate this more clearly. FABP type A is located predominantly in the proximal-tubular system, while FABP type B is mainly found in the distal-tubular system. The epithelial cells of the proximal convoluted tubules, may, however, contain both types of FABP since they are also moderately reactive with anti-(FABP type B) antiserum. More evidence is necessary to prove if these cells are comparable with intestinal cells, which contain two types of FABP and, in addition, in the ileum a FABP counterpart, gastropin [18,50]. This is interesting since both cell types are highly polarized absorptive cells with well-developed brush borders. Western-blot analysis of cytosolic protein from rat glomeruli and tubules showed that the specific kidney FABP is predominantly located in the proximal tubule [51]. In human kidney-specific staining with anti-(human liver FABP) antibodies was confined to cortical-tubular cells [9].

Concerning the functional significance of the two types of FABP in different cells of the kidney, we can only speculate. Metabolic functions are unevenly distributed along the

nephron. In the proximal tubules, fatty acids serve as energy supply. These cells have a high capacity for gluconeogenesis, but a low glycolytic capacity [27]. Depending on the serum concentration, the excess of fatty acids is incorporated in triacylglycerols [26,28]. In the distal part of the nephron triacylglycerol synthesis is low, gluconeogenesis is absent, but the capacity of glycolytic enzymes is much higher. In proximal tubules both mitochondrial and peroxisomal ß-oxidation of fatty acids take place, whereas in the distal tubules only mitochondrial oxidation occurs [26,52,53]. Proximal and distal tubules also differ in re-absorption and excretion processes [30]. In proximal tubules, a large variety of substrates are reabsorbed; in distal tubules they are predominantly electrolytes. In this respect the differences in ligand specificity of the FABP types may be important. The FABP type A may similarly to liver FABP, act as a rather general carrier for hydrophobic compounds, whereas the B type is specific for fatty acid transport. Thus, the regional distribution of the different types of renal FABP may be related to differences in fatty acid metabolism and/or in resorption or excretion processes.

In conclusion, we isolated two FABP types from human renal cortex with different characteristics and distribution. FABP type B seems related to, and shows identity with heart FABP. FABP type A resembles liver FABP in many respects. The differences in isoelectric point and amino acid composition, however, indicate that it might be a kidney-specific FABP type. Definitive evidence about the structure of both FABPs has to await analysis of their amino acid and/or cDNA sequence.

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CHAPTER 4

MOLECULAR IDENTIFICATION OF THE LIVER- AND THE HEART-TYPE FATTY ACID-BINDING PROTEINS IN HUMAN AND RAT KIDNEY

Use of the reverse transcriptase polymerase chain reaction

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SUMMARY

The cDNAs of two types of fatty acid-binding protein (FABP) present in human kidney, previously described as types A and B, were isolated using reverse transcriptase-PCR (RT-PCR) with human kidney mRNA and various sets of primers. The cDNA fragments were cloned and sequenced. Renal FABP type A and B cDNA appeared to be completely identical with human liver- and heart-type FABP cDNA, respectively.

In the second part of this study we demonstrated the presence of liver type FABP in rat kidney by chromatography, e.l.i.s.a. and immunocytochemistry. The ratio and cellular distribution of the two FABP types varies markedly in human and rat kidney. Using RT-PCR we were also able to prepare and identify liver and heart type FABP cDNAs with mRNA from both male and female rat kidney.

INTRODUCTION

In mammalian cells, fatty acids are important molecules for energy delivery and for synthesis of membrane lipids and lipid mediators. Fatty acids are metabolized in mitochondria, peroxisomes and on the endoplasmic reticulum. The transport of fatty acids from the plasma membrane to these cellular organelles is believed to be performed by fatty acid-binding proteins (FABPs) [1-3]. On the basis of their primary structure at least five FABPs, with sequence similarities of 25-65%, have been identified [3]. These FABPs have been named, liver, heart, intestinal, adipocyte and myelin FABP, after the tissue from which they were initially isolated. The existence of different FABP types suggests a type-specific function. The presence of more than one FABP type in a tissue e.g. intestine [4], stomach [5] and kidney [6,7] supports this hypothesis. Since the first isolation of an FABP cDNA, the rat liver FABP cDNA [8], cDNAs of all five FABP types have been obtained [9-16].

In rat kidney two FABP types have been demonstrated. One was identified as heart type FABP, based on biochemical and immunological characterization [8,17,18] and mRNA detection [11,19]. The second type was biochemically and immunologically different from heart- or liver-type FABP and was named renal-specific FABP [6,18,20]. Amino acid sequencing, however, demonstrated that this renal-type FABP was identical with α_{2U}-globulin [18,21]. The latter protein is a secretory protein of the liver and undergoes endocytotic uptake into the proximal tubules [22]. The α_{2U}-globulin molecule is structurally not a FABP but is more closely related to the lipocalins as lactoglobulin and serum retinol-binding protein [23,24]. Recently we isolated two FABP types from human kidney; one (type A) showed similarity with human liver FABP and the other (type B) with human heart FABP [7]. However, cDNA analysis has yet to prove the exact identity of both human kidney FABPs.

In the present study we describe the preparation and identification of the two human renal FABP cDNA types by reverse transcriptase-PCR (RT-PCR). We also applied this PCR-technique to investigate the FABP types of rat kidney, since we obtained evidence indicating the presence of liver type FABP in this tissue.

MATERIALS AND METHODS

Materials

Moloney Murine Leukemia virus [MMuLV] RNase H$^-$ reverse transcriptase was obtained from Bethesda Research Laboratories, Life Technologies, Gaithersburg, MD, USA; recombinant Taq DNA polymerase Amplitaq from Perkin-Elmer Cetus, Norwalk, CT, USA; Sequenase version 2.0 from United States Biochemical, Cleveland, OH, USA; synthetic oligonucleotide primers from Pharmacia, Uppsala, Sweden; wheat germ extract, L-[^{35}S]methionine, [α-^{35}S]-dATP [15 mCi/ml], [α-^{32}P]-dATP [10 mCi/ml] from Amersham Int, Little Chalfont, Bucks., U.K.; SP6-RNA polymerase, RNasin and pGEM-5 Zf[+] from Promega Corporation, Madison, NY, USA; peroxidase-conjugated goat anti-(rabbit IgG) from Tago, Burlingama, CA, USA; rabbit peroxidase anti-peroxidase couples were from Dakopatts, Glostrup, Denmark; goat anti-(Tamm-Horsfall glycoprotein) from Cappel, West Chester, PA, USA.

Human kidney tissue was obtained after nephrectomy. Rat kidneys were from 12-week-old Wistar rats.

Methods

RNA isolation and blot hybridization

Total RNA was extracted with LiCl/urea by a modification of the procedure of Auffray et al. [25]. Kidney tissue (1-2 g) was homogenized in a mixture of 3 M-LiCl/6 M-urea/20 mM-sodium acetate (pH 5.2)/heparin (2 mg/ml) and left overnight at 0°C. The RNA precipitate was pelleted by centrifugation for 15 min at 16,000 g and washed once with 4 M-LiCl/8 M-urea. After centrifugation for 15 min at 16,000 g the pellet was dissolved in 50 mM-sodium acetate (pH 5.2)/0.2 % SDS/2 mM-EDTA and extracted successively with phenol, phenol/chloroform and chloroform. Subsequently the RNA was precipitated with ethanol and stored at - 20°C. Poly-(A)$^+$-RNA was selected on oligo(dT)-cellulose [26].

For Northern blot analysis, RNA was electrophoresed on 1% agarose gels in the presence of formaldehyde. Prior to electrophoresis, ethidium bromide was added to the RNA samples in order to allow visualization of the rRNAs in the gel. In this way, it was ascertained that the amounts of RNA in the different lanes were approximately the same. Following electrophoresis the RNA was transferred to nitrocellulose filters [26]. DNA probes were labelled by the random priming method [27]. The RNA blot was hybridized at 37°C in 0.5 M-sodium phosphate as described by Church & Gilbert[28]. The blots were finally washed in 0.05 M-sodium phosphate at 65°C, dried and subjected to autoradiography at -70°C using intensifying screens.

RT-PCR-procedure

Primers derived from the heart and liver FABP cDNA sequence were designed so as to amplify the coding region. The primers used are given in Table 1.

Table 1. Primers used in RT-PCR

Primer	Sequence
1. Human heart FABP (forward)	GCCAGCATCACCATGGTGGACGCTTTC
2. Human heart FABP (reverse)	ATCACCAGTGGATCCAGGTCATGCCTC
3. Rat heart FABP (forward)	TCTCATTGCACCATGGCGGACGCCTTT
4. Rat heart FABP (reverse)	AGTGACGGGGGATCCAGGTCACGCCTCCTT
5. Human liver FABP (forward)	AAGGGGGTGTCGGAAATCGTG
6. Human liver FABP (forward)	ATTGCCCATATGAGTTTCTCCGGCAAGTAC
7. Human liver FABP (reverse)	GCGCTGCAGGGATCCGTCGAC[T]$_{17}$
8. Human liver FABP (reverse)	AATGGGATCCTGTTTAAATTCTCTTGCTGATTCT
9. Rat liver FABP (forward)	GCCCATATGAACTTCTCCGGCAAGTAC
10. Rat liver FABP (reverse)	CTGGGATCCCTAAATTCTCTTGCTGACTCTCTT

First strand synthesis reactions were carried out with 50 μg total RNA or 2 μg poly-A$^+$ RNA in a total volume of 20 μl containing 50 mM-Tris/HCl (pH 8.3)/75 mM-KCl/3 mM-$MgCl_2$/125 μM-dNTPs/40 units RNasin/0.5 μg primer and 400 units M-MuLV reverse transcriptase. A reverse primer was added to the RNA sample, heated for 10 min. at 70°C and quenched on ice. The reverse transcription reaction was carried out for 1 hr at 37°C and 30 min at 45°C.

The PCR amplification reactions were carried out in a total volume of 100 μl containing 2 μl of the reverse transcription reaction, 0.5 μg of the reverse and forward primer, 10 mM-Tris/HCl (pH 8.3)/50 mM-KCl/1.5 mM-$MgCl_2$/0.001 % gelatin/250 μM-dNTPs and 2.5 units Amplitaq. The mixture was overlaid with mineral oil. After 8 min denaturation at 94°C, 40 cycles of amplification were carried out by using a step program (94°C, 1 min; 50°C, 1.5 min; 72°C, 1.5 min) followed by a 2 min final extension at 72°C.

Cloning and sequencing of the cDNAs

RT-PCR products were digested with restriction enzymes which recognize specific sites in the primers. The cDNA fragments were cloned in M13mp18 and PGEM 5Zf(+). Plasmids with cDNA inserts were single and double strand sequenced in both directions with Sequenase, using the supplier's recommendations.

In vitro transcription/translation assay

The cDNA insert was ligated in PGEM 5Zf(+), as described above, grown in JM101 and afterwards linearized with BamHI. RNA transcripts were synthesized in vitro from the BamHI digested clone in the presence of SP6 RNA polymerase and the dinucleotide primer G-(5')ppp(5')G [29]. In vitro translation in a nuclease-treated wheat germ extract was performed for 60 min at 25°C in 30 μl of a reaction mixture, containing 1 mM-amino acids/1 mM-[^{35}S]methionine and 0.65 M-KCl.

Immunoprecipitation of newly synthesized FABP

200 μl Protein-A Sepharose CL-4B in phosphate-buffered saline (10%, v/v) was incubated with 75 μl anti-(human liver FABP) serum for 2 h at room temperature under rotation. Non-specifically bound proteins were removed by washing the gel suspension 4 times with 500 μl buffer A (500 mM-NaCl/10 mM-Tris/HCl (pH 7.4)/ 0.05% Nonidet P40). Subsequently, the gel suspension was incubated with 25 μl of the translation assay mix in 500 μl 150 mM-NaCl/50 mM-Tris/HCl (pH 7.4)/0.05% Nonidet P40 for 3 h at 4°C, under rotation. Finally, the gel suspension was washed 4 times with buffer A and 50 μl of SDS gel electrophoresis loading buffer was added. SDS gel electrophoresis was performed on a 15% polyacrylamide gel.

Immunocytochemistry

Kidney tissue from male and female Wistar rats (200 g) were fixed for 2 h in 2% periodate-lysine-paraformaldehyde and embedded in paraffin. Deparaffinized sections were treated with 1% (v/v) H_2O_2 in methanol in order to remove intrinsic peroxidase activity. After rehydration and treatment with 20% (v/v) normal goat serum in Tris-buffered physiological saline for 30 min, sections were incubated with rabbit antisera raised against rat liver FABP or rat heart FABP, optimal dilutions being 1:25 - 1:50. Immunopositive sites were detected by subsequent incubations with goat anti-(rabbit IgG-peroxidase) and rabbit peroxidase anti-peroxidase complex. Peroxidase activity was visualized using diaminobenzidine as substrate. Sections were counterstained for 1 min with Harris's heamatoxylin and embedded in glycerol/gelatine mounting medium. Preimmune sera and sera raised against unrelated antigens were used as controls. In addition, immune sera were absorbed (18 h, 20°C) with both FABPs, the optimal amount being 25 μg of FABP/100 μl diluted serum. Antiserum against Tamm-Horsfall glycoprotein was used for the localization of distal tubules.

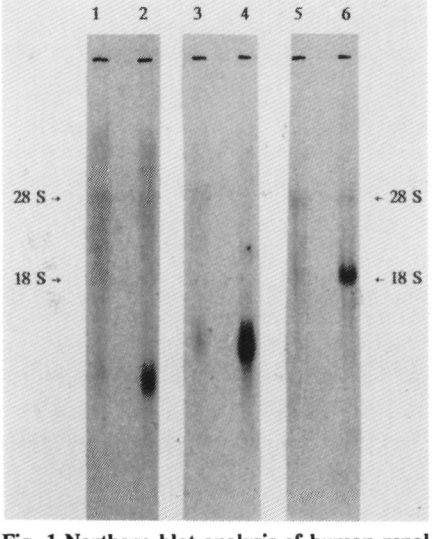

Fig. 1 Northern blot analysis of human renal FABP type A and B transcripts. Total RNA (50 μg) (lanes 1, 3, 5) and poly-(A)$^+$ RNA (20 μg) (lanes 2, 4, 6) from human renal tissue was fractionated by electrophoresis on 1% agarose-formaldehyde denaturing gels, transferred to nitrocellulose and hybridized with random-primed labelled human liver FABP cDNA (lanes 1 and 2), human heart FABP cDNA (lanes 3 and 4) or human actin cDNA (lanes 5 and 6).

Other procedures

Preparation of rat kidney cytosol, Sephacryl S-200 gel filtration and DEAE-Sepharose chromatography were performed as described for human kidney [7]. The FABP contents of kidney cytosol were determined in e.l.i.s.a. as described [17].

Rat liver and heart FABPs were isolated as described [30,31] and antisera were raised in rabbits [17,32]. cDNA probes of human heart (muscle) FABP and of human liver FABP were prepared as described previously [15] or during this study respectively.

RESULTS AND DISCUSSION

Identification of two FABP transcripts in human kidney.

Northern blot analysis of total RNA (50 μg) and poly(A)$^+$-mRNA (20 μg) from human kidney showed the presence of two transcripts using human liver and heart FABP cDNA as probe. The transcripts were 0.5 and 0.7 kb in length for the liver and heart types, respectively (Fig. 1). From the autoradiogram it can be concluded that the mRNA concentration of both FABP types is rather low in kidney, since 50 μg of total RNA only gave a faint band at the position of the FABP transcripts.

Isolation of cDNAs of two FABP types of human kidney

After the identification of two transcripts, we attempted the isolation of both cDNAs, by RT-PCR using primers based on the cDNA sequences of human liver- and heart-type FABP cDNAs. The structural and immunological data concerning the FABP type A from human kidney revealed many similarities but also some differences compared with human liver FABP [7]. The cDNA sequence was required to establish the structure. For the isolation of the FABP type A cDNA the RACE (rapid amplification of cDNA ends) protocol described by Frohman et al. [33] was followed. The reverse transcription primer (primer 7) was an oligonucleotide with 17 dT residues and an adaptor sequence containing three restriction enzyme recognition sites. The forward primer (primer 5) was derived from human liver FABP cDNA (nucleotides 106-126) and is located in a highly conserved region [10]. The DNA fragment isolated after RT-PCR was cloned in M13mp18 and sequenced. This 350 bp

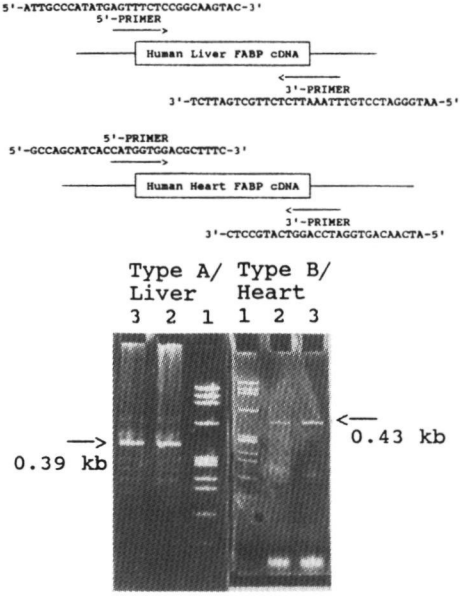

Fig. 2 Reverse Transcriptase PCR isolation of human kidney FABP type A and B cDNA. Poly-(A)$^+$ RNA (2 μg) was reverse transcribed with 3'-primers of human liver or heart FABP cDNA, amplified in PCR using 5'- and 3'-primers and subjected to polyacrylamide mini-gel electrophoresis. Lane 1 shows the ΦX174 x Hae III marker and lane 2 and 3 contain the RT-PCR fragment prepared with 1 and 2 μg of poly(A$^+$) RNA respectively.

fragment appeared to be completely identical with human liver FABP cDNA. Subsequently RT-PCR was applied with a reverse and forward primer (primers 8 and 6) encoding the 3'- and 5'-ends respectively of the human liver FABP cDNA coding region, respectively (Fig. 2). The 403 bp fragment was isolated and cloned in pGEM-5 Zf(+) and sequenced. The sequence appeared to be identical with the liver FABP cDNA sequence described by Chan et al. [9].

Data on the protein have indicated that the FABP type B from human kidney is identical with the heart type FABP [7]. To establish this, we carried out a reverse transcription with primer 2, which encoded the 3'-end of the coding region of the heart FABP cDNA. The reverse transcription mixture was amplified with the same reverse primer and primer 1, encoding the 5'-end of the heart FABP cDNA coding region (Fig. 2). A DNA fragment of 432 bp was isolated, digested with restriction enzymes, which recognize sites present in the primers, and subcloned in M13mp18. The sequence appeared to be fully identical with the heart- or muscle-type FABP cDNA sequence published by Peeters et al. [15].

Both total RNA and mRNA from both males and females could be used for the isolation of the renal FABP cDNAs. The FABP mRNA concentrations appeared to be similar when RT-PCR was applied with equal amounts of RNA of male or female kidney.

In vitro transcription/translation of human renal type A FABP cDNA

The identity of the cDNA fragment isolated with human liver FABP cDNA primers was confirmed by an in vitro transcription/translation assay followed by immunoprecipitation. The pGEM-5 Zf-(+), containing the human renal FABP type A cDNA, was linearized with Bam HI, which is located 5 bp downstream of the stop codon. After linearization, the pGEM-5Zf(+)-FABP type A vector was used in an in vitro transcription assay. The RNA product yielded in an in vitro translation assay the 14.3 kDa product (Fig. 3). The smaller products are due to a start of translation at ATG codons located further downstream. These ATG codons correspond with amino acid positions 19 and 74 [9,10]. Immunoprecipitation of the translation product with antibodies against human liver FABP gave the same result (Fig. 3).

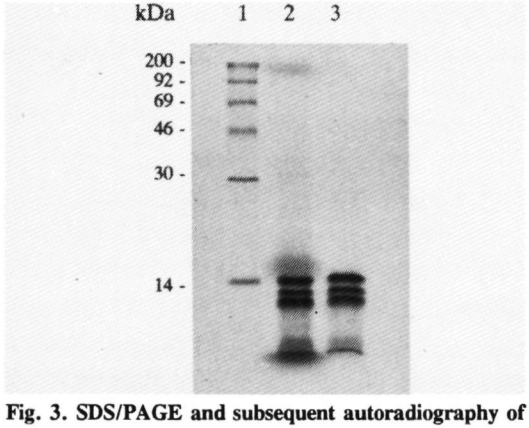

Fig. 3. SDS/PAGE and subsequent autoradiography of the products obtained after in vitro transcription/translation of human liver FABP cDNA. pGEM 5 Zf(+)-HL-FABP was Bam HI digested, transcribed with SP6-RNA polymerase and translated with wheat germ extract. The translation product was immunoprecipitated with antibodies against human liver FABP. Lane 1, molecular-mass markers; lane 2, total translation mixture (4 μl); lane 3, an equal amount of total translation mixture after immunoprecipitation (15 μl).

Renal FABP type B cDNA was directly cloned in the expression vector pET8c. After expression in *Escerichia coli* the protein was identical with heart (muscle) FABP on the basis of various

biochemical and immunological tests (results not shown) [15].

Identification of the FABP types and FABP RNA transcripts in rat kidney

As already mentioned, male rat kidney contains the heart-type FABP and α_{2U}globulin, female kidney only contains the heart-type FABP [6,17,18,20]. The presence of liver FABP in rat kidney has never been demonstrated. We obtained evidence that the liver-type FABP is also present in rat kidney, as in human kidney. The low-molecular-mass fraction obtained by Sephacryl gel filtration of rat kidney cytosol revealed on DEAE-Sepharose chromatography two fractions with fatty acid-binding activity. The first fraction was not bound, like the liver-type FABP, whereas the second fraction was eluted from the column during the gradient, like the heart-type FABP. The first peak bound about 10% of the [^{14}C]oleic acid bound by the second peak.

E.l.i.s.a. showed low amounts of liver-type FABP in rat kidney cytosol (Table 2). The concentrations are much lower than those of the heart-type FABP and the ratio of liver- and heart-type FABP differs considerably from that in man.

Table 2 FABP content of kidney. Values (determined by e.l.i.s.a.) are means ± S.D. for five or six individuals.

	Content (nmol/mg of cytosolic protein)	
	Liver-type	Heart-type
Rat male	0.078 ± 0.0005	0.79 ± 0.10
Rat female	0.051 ± 0.0007	0.67 ± 0.04
Human male	0.63 ± 0.190	0.38 ± 0.26

Immunocytochemistry on rat kidney sections also established the presence of liver-type FABP. Using anti-(liver FABP) anti-serum, proximal as well as distal tubules reacted positively, the latter being more immunoreactive (Fig 4a). In addition, the collecting tubules and the papillary epithelium are also immunoreactive but glomeruli did not stain. Preabsorption of immune serum with liver FABP resulted in a complete abolition of staining (Fig. 4b). No difference could be observed between male and female rat kidneys. Distal tubules were the only structures strongly positive when stained with anti-(heart FABP) antiserum (Fig 5): proximal tubules were not distinctively positive and glomeruli were negative. Preabsorption of immune serum with liver FABP had no effect. Male and female rat kidneys reacted similarly. The cellular distribution of the heart type is similar in rat kidney to that previously found in human kidney [7]. The liver-type FABP, however, is restricted to the proximal convoluted and straight tubules in human kidney [7]. Like the kidney, the rat stomach also shows the presence of both heart- and liver-type FABP with a specific cellular distribution [5].

With RT-PCR we also investigated the presence of the transcripts of liver- and heart-type FABP in rat kidney. Poly(A)$^+$ RNA was isolated and reverse-transcribed with a reverse primer (primer 4 or 10), which partially overlapped the 3'-end of the coding region of the rat heart- or liver-type FABP cDNA respectively. PCR amplification was accomplished by using the reverse primer and the appropriate forward primer (primer 3 or 9), which partially overlapped the 5'-end of the coding region of the FABP cDNA. Subsequently the RT-PCR products were sub-

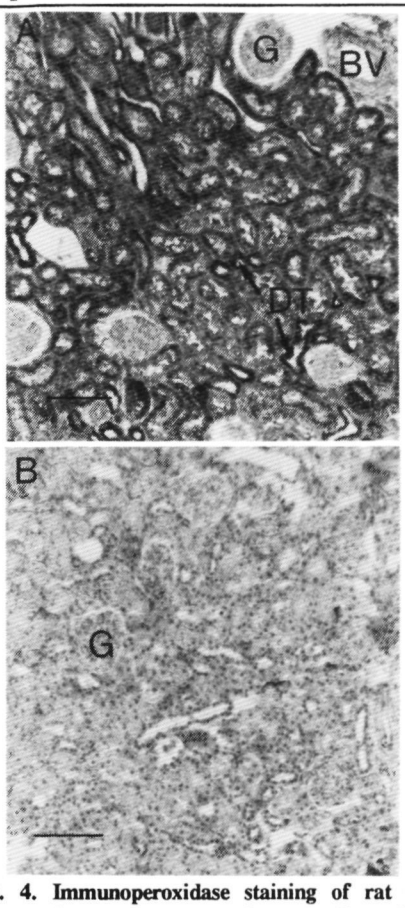

Fig. 4. Immunoperoxidase staining of rat renal tissue using antiserum against rat liver FABP before (a) and after (b) preabsorption with rat liver FABP. Proximal tubules (PT) and distal tubules (DT) are positive, the latter being more immunoreactive. Glomeruli (G) and blood vessels (BV) are negative. The bar represents 100 μm.

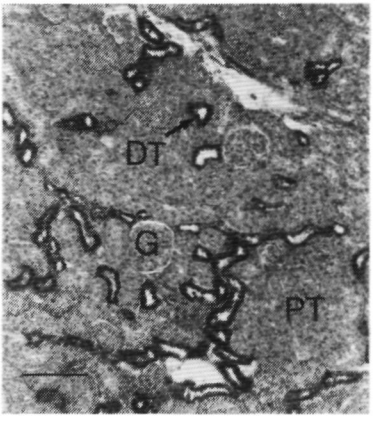

Fig. 5. Immunoperoxidase staining of rat renal tissue using antiserum against rat heart FABP. Distal tubules (DT) are strongly immunopositive. PT, proximal tubules; G, glomeruli. The bar represents 100 μm.

jected to polyacrylamide mini-gel electrophoresis and electroblotting to Hybond N^+. The DNA blots were probed with human heart and liver FABP cDNA. As a control RT-PCR was also applied on rat heart and liver mRNAs with both rat liver and heart FABP type cDNA primers.

The rat heart FABP cDNA could be demonstrated on the blot with the human muscle FABP cDNA probe, to be present in rat heart and kidney mRNA, but not in rat liver mRNA (Fig. 6). The blot hybridized with human liver FABP cDNA showed the presence of liver FABP mRNA in both liver and kidney mRNA, but not in heart mRNA (Fig. 6). Bands at higher molecular masses may be due to reverse transcription of pre-mRNAs. Based on the RT-PCR and hybridization results, the content of the mRNAs of the liver and heart FABP types do not differ markedly in kidneys of male and female rats.

Studies on transgenic animals expressing reporter genes under the control of the liver FABP gene promoter have revealed that expression of liver type FABP is possible in mouse kidney [34,35]. The heart-type FABP mRNA has been demonstrated in rat kidney by others [11,19] Its content shows marked variations from fetal to adult life [19]. Concerning the physiological relevance of the two FABP types in kidney we can only speculate. The liver-type FABP binds various ligands and may be involved in the renal excretion of exogenous and endogenous metabolites. The liver-type FABP also binds some drugs [2,3] and may in this way prevent

nephrotoxicity. The heart-type FABP only binds fatty acids and seems to be involved in lipid metabolism.

In conclusion, we have isolated the cDNAs of both human renal FABP types by RT-PCR. Human renal FABP type A and B cDNA appeared to be fully identical with human liver and heart FABP cDNA respectively. The cDNA analysis gave the conclusive evidence for the presence of liver and heart type FABP in human kidney, in agreement with most or all of the physiochemical and immunochemical data on the protein [7]. Furthermore, we also demonstrated the presence of liver and heart FABP types and their mRNAs in kidneys from both male and female rats. The significance of the occurrence in kidney of two FABP types with different ligand specificities and cellular distributions requires further investigation. The availability of cDNAs and FABP preparations of both types from human and rat kidney will allow future work in this area.

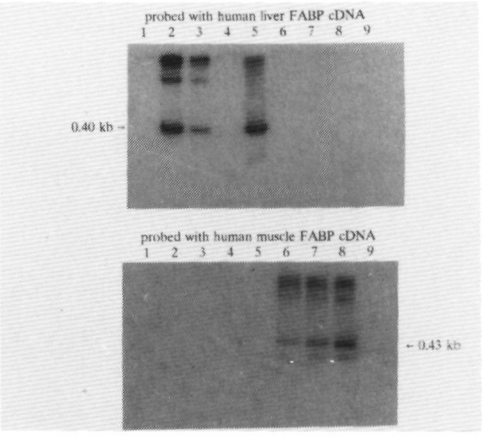

Fig. 6 Identification of the FABP RNA transcripts in rat tissues. Poly(A)$^+$ RNA (2 μg) from rat tissues including male kidney (lanes 2, 6), female kidney (lanes 3, 7), heart (lanes 4, 8) and liver (lanes 5, 9) were reverse transcribed with 3'-primers of rat liver-type FABP cDNA (lane 2-5) and rat heart-type FABP cDNA (lanes 6-9), respectively. The reverse transcription mixture was amplified in PCR and subjected to polyacrylamide mini-gel electrophoresis, electroblotted to Hybond N$^+$ and probed with human liver and heart FABP cDNA, respectively. Lane 1 shows the ΦX x HaeIII marker.

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CHAPTER 5

EXPRESSION OF HUMAN LIVER FATTY ACID-BINDING PROTEIN IN *ESCHERICHIA COLI* AND COMPARATIVE ANALYSIS OF ITS BINDING CHARACTERISTICS WITH MUSCLE FATTY ACID-BINDING PROTEIN

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Biochim. Biophys. Acta submitted

SUMMARY

Human liver fatty acid-binding protein (L-FABP) has been efficiently expressed in *Escherichia coli*. The cDNA encoding human liver FABP was under control of the T7 RNA polymerase promoter in the expression vector pET-3b. Expression required overnight induction with isopropyl ß-D-thiogalactopyranoside in the presence of the bacterial RNA polymerase inhibitor, rifampicin. The protein could be purified by $(NH_4)_2SO_4$ fractionation, anion-exchange and gel filtration chromatography and was recognized by anti-(human L-FABP) antiserum.

The binding characteristics of delipidated recombinant human L-FABP and muscle FABP (M-FABP) for fatty acids of different chain-length and saturation grade and for various hydrophobic ligands were determined by radiochemical analysis and for L-FABP also by fluorescence. The apparent binding affinity of the ligands was calculated by using displacement curves of oleic acid and dansylamino-undecanoic acid (DAUDA). L-FABP showed a preference for binding of long-chain saturated and unsaturated fatty acids up to $C_{24:1}$ whereas the M-FABP has a preference for unsaturated fatty acids, especially with 18 C atoms. L-FABP also binds palmitoyl derivatives and many other hydrophobic ligands, generally however with a lower affinity than fatty acids. M-FABP binds besides fatty acids only oestradiol and testosterone with high affinity. Fatty acids with fluorescent reporter groups are also more tightly bound by L-FABP. A direct assay and displacement studies of oleic acid gave the same K_d value of DAUDA for L-FABP. Fluorescence enhancement and displacement studies indicate that the binding of fluorescent fatty acids is determined by both the fluorescent reporter group and the carbon chain.

INTRODUCTION

All living cells utilize fatty acids as source of energy and/or for synthesis of membrane lipids and lipid mediators. Fatty acids may modulate various cellular processes by their interaction with enzymes, membranes, ion channels, receptors or genes. Utilization by cellular organelles requires facilitation of their solubility and transport, in which fatty acid-binding proteins (FABPs) are supposed to be involved. This group of low-molecular mass proteins with high sequence similarity is known to consist of seven different types at the moment [1-5].

Recently we isolated two types of FABPs from human kidney, which showed similar characteristics as L-FABP and M-FABP on base of molecular mass, isoelectric point, binding affinity for oleic acid, immunological crossreactivity and amino acid content [6]. Isolation of both cDNAs for these FABPs by reverse transcriptase PCR on kidney mRNA established the presence of L-FABP and M-FABP in human kidney [7]. The two types are differently distributed, L-FABP is present in the proximal tubules whereas M-FABP is predominantly present in the distal tubules [6].

M-FABP was expressed in E.coli [8], crystallized and its three-dimensional structure determined [9]. The structure of human M-FABP was found to be similar to that of other members of the FABP family; containing ten anti-parallel ß-strands arranged in two orthogonal ß-sheets and two short α-helices located between the first and second ß-strand [9]. In spite of

their high structural similarity, intestinal, adipocyte, myelin and muscle FABP types bind their fatty acid differently. The muscle and myelin FABP types bind the fatty acid in an U-shape [9,10] whereas the intestinal and adipocyte FABP types bind the fatty acid in an more extended conformation [11-14]. Amino acids involved with fatty acid binding are different between muscle and intestinal FABP [15].

The L-FABP, the other type present in human kidney, has not been expressed in *E. coli* until now. However the cDNA encoding rat L-FABP was expressed in *E. coli* and amounts of recombinant protein could be obtained [16,17]. Attempts to analyze the three-dimensional structure of the L-FABP failed thusfar [18]. Analysis of the binding characteristics revealed that the fluorescent probe DAUDA is bound by rat L-FABP. Binding is accompanied by a shift in wavelength of the fluorescence emission maximum and a large fluorescence enhancement [19]. The amount of L-FABP in various tissues could be calculated from the fluorescence signal [20]. Displacement of DAUDA could be determined for various ligands which revealed information about the binding characteristics and binding pocket of L-FABP [21]. Binding studies with fluorescent anthroyloxy-labeled fatty acids suggested that the binding pocket of L-FABP is more hydrophobic compared to M-FABP and adipocyte FABP [22-24]. However investigations with the fluorescent trinitrophenyladenosine-triphosphate [25] suggest that the fluorescent group is involved in binding rather than the fatty acid.

The presence of two FABP types in human kidney [6,7] suggests a specific function and difference in binding characteristics of both proteins. Several independent binding studies have been carried out with L-FABP or M-FABP with fatty acids and some other hydrophobic compounds [for refs see 5 and 26], but simultaneous comparative studies of binding charcteristics of both FABP types lack. Moreover, most studies have been carried out without delipidation of the protein and with isolated tissue preparations, which may contain contaminating other lipid-binding proteins. Therefore we expressed for the first time the human L-FABP in *E. coli* and did extensive studies with delipidated recombinant L-FABP and M-FABP to analyse their binding affinities for various types of fatty acids and a large series of hydrophobic ligands. We used the radiochemical Lipidex procedure in combination with an equivalent competition method based on a newly developed procedure for analysis of ligand-protein binding data [27]. We also compared the interaction of various fluorescent fatty acids with both FABP types and determined the binding affinity of DAUDA for L-FABP by an analogous method [28]. For comparison with the radiochemical data we studied also the displacement of DAUDA from L-FABP by various fatty acids and hydrophobic ligands and calculated the apparent K_i values for these ligands, using a newly described mathematical approach.

MATERIALS AND METHODS

Materials

Oligonucleotide primers and the FPLC Hiload Q Sepharose column were obtained from Pharmacia Biotechnologies Int., Uppsala, Sweden; rifampicin and saturated and unsaturated

fatty acids from Sigma Chemical Co., St. Louis, MO., U.S.A.; IPTG from Life Technologies Inc., Gaithersburg, MD, U.S.A.; Lipidex 1000 from Canberra-Packard, Groningen, The Netherlands; 11-dansylamino-undecanoic acid, 2-(9-anthroyloxy)palmitic acid, 12-(9-anthroyloxy)oleic acid and 16-(9-anthroyloxy)palmitic acid were from Molecular Probes, Junction city, OR., U.S.A. and [1-C^{14}]oleic acid from Amersham International, Little Chalford, U.K.. All other reagents were of analytical grade.

Methods

Expression and purification of recombinant human liver FABP.

Cloning of human liver FABP cDNA in expression vector pET-3b.

Two single stranded complementary oligonucleotides homologous to the first 10 amino acid residues of human L-FABP with a NdeI-site at the start codon were synthesized: 5'-TATGAGTTTCTCCGGCAAGTACCAACTGCA-3' and 3'-ACTCAAAGAGGCCGTTCATGGTTG-5'. The oligonucleotides were purified by fast liquid chromatography. The human L-FABP cDNA isolated by us with reverse transcriptase PCR [7] was digested with PstI and BamHI. The single stranded oligonucleotides were hybridized and ligated in a 10-fold molar excess with the PstI/BamHI partial human L-FABP cDNA fragment to create a NdeI site at the L-FABP cDNA. The nucleotide sequence of the FABP cDNA after primer ligation was verified using the dideoxy chain termination technique. The NdeI/BamHI L-FABP cDNA was ligated in the similar sites of the expression vector pET-3b (Fig. 1).

Expression of L-FABP in *E. coli*

The pET-3b/L-FABP construct was used to transform *E. coli* K12 strain BL21(DE3) pLys S [29]. Transformed cells were grown overnight on 2xTY plates containing 100 μg/ml ampicillin and 35 μg/ml chloramphenicol (2xTY/AC) [30]. A 80 ml overnight culture was grown from a freshly streaked out plate and 5 ml was used to inoculate 1.5 l 2xTY/AC in a 2 l flask. These were incubated at 37°C until the cell density reached an A_{600} of 0.8-1.0. Expression by T7-RNA polymerase was induced by addition of 0.5 mM-IPTG and incubation for 45 min at 37°C. Subsequently 175 μg/ml rifampicin was added to the culture and incubation continued for 16 h at 37°C. To improve expression, other media, TB and SBLH [30] were also evaluated.

Purification of recombinant L-FABP

The cells were harvested by centrifugation for 20 min at 5,000 g. The pellets were washed once with PBS, stored at -70°C to facilitate cell lysis and resuspended in 10 mM-Tris/HCl (pH 8.5)/0.01% sodium azide. The cells were passed through a French press cell and centrifuged for 30 min at 30,000 g. The supernatant was saturated with ammonium sulphate in two steps, the first step to 50% and the second to 70% ammonium sulphate. After each

saturation step the mixture was centrifuged for 30 min at 30,000 g and the ultimate supernatant was dialyzed for 48 h against 10 mM-Tris/HCl (pH 8.5)/0.01% sodium azide. The dialysate was bound to a FPLC Hiload Q-Sepharose column and eluted with a gradient of 10 to 100 mM-Tris/HCl (pH 8.5). Recombinant L-FABP was purified from DNA traces by gel filtration on Sephadex G-50 (1.5cm x 120cm). Immediately before binding assays, FABP preparations were delipidated with the Lipidex procedure [31].

Fatty acid-binding assays with oleic acid

All binding assays were carried out in a volume of 0.5 ml 10 mM-Tris/HCl (pH 8.0)/ 1% ethanol at 37 °C. K_d values for the binding of recombinant human L-FABP and M-FABP to [1-^{14}C]oleic acid were determined with the Lipidex procedure and Scatchard analysis [31]. The K_i values for binding of other fatty acids and hydrophobic ligands to FABP were determined by displacement analysis according to the method of van Zoelen [27]. Displacement curves were made with the Lipidex-procedure by addition of 1 μM-[1-^{14}C]oleic acid in the presence of unlabeled oleic acid (0-10 μM) to 0.2 μM-L-FABP or 0.28 μM-M-FABP. Subsequently the displacement percentage of [1-^{14}C]oleic acid by various hydrophobic ligands was analyzed by addition of 1 μM-[1-^{14}C]oleic acid and 1 μM-competitor to the same amount of FABP. From the displacement curve the concentration of unlabeled oleic acid was read which is needed to obtain the same percentage of displacement as with 1 μM-competitor. Van Zoelen [27] showed that the K_i value of the competitor can be calculated with the formula:

$$\frac{L_i - L_s^u}{L_s^u\,(1\text{-}cpm^{bound}/cpm^{added})} = \frac{K_i - K_d}{K_d}$$

In this equation L_i is the added concentration of competitor, L_s^u is the concentration of non-labeled oleic acid required to obtain the same percentage of displacement as found with L_i, K_i the dissociation constant for the competitor and K_d the dissociation constant for oleic acid. Van Zoelen [27] showed that if competition is complete a correction term (1 - cpm^{added}/cpm^{bound}) is included to account for the fact that the free ligand concentration can be significantly lower than the ligand concentration added.

Fatty acid-binding assays with fluorescent ligands

Fluorescence enhancement of various fluorescent fatty acids with recombinant L-FABP and M-FABP.

All fluorescence experiments were carried out at a Shimadzu-500 fluorometer. The fluorescence was analyzed for 1.0 μM-fluorescent fatty acid in the absence or presence of 2.0 μM-L-FABP or 2.8 μM-M-FABP in 1 ml 10 mM Tris/HCl (pH 8.0)/1% ethanol at 25 °C. The emission spectrum between 480 and 550nm for DAUDA was determined at an excitation wavelength of 328 nm and for DAUDA in the presence of FABP at 345 nm. Maximum fluorescence of the other ligands with or without protein was obtained by scanning between 380

and 550 nm at the excitation wavelength indicated.

Displacement studies with DAUDA

All experiments were carried out in a volume of 1.0 ml 10 mM Tris/HCl (pH 8.0)/1% ethanol at 25 °C. Displacement studies with DAUDA were performed by addition of 1 μM-DAUDA with or without 1 μM-competitor to 0.2 μM-L-FABP. Emission spectra (480nm-550nm) were scanned at an excitation wavelength of 345 nm because the various competitors cause a different wavelength shift of the fluorescence emission maximum. The displacement percentage could be calculated with the equation:

$$\frac{F_i - F_0}{F_{max} - F_0} \times 100$$

In this equation F_i is the fluorescence of DAUDA in the presence of FABP and competitor at the wavelength of maximal emission, F_0 is the fluorescence of DAUDA without protein at 545 nm and F_{max} is the fluorescence of DAUDA in the presence of FABP at 505 nm.

To determine the K_d for DAUDA saturation curves were made by analysis of the fluorescence of 0.2 μM L-FABP with increasing concentrations of DAUDA (0-5 μM). Values were corrected for the fluorescence of the DAUDA solution itself. The saturation curves were converted mathematically into a displacement type of curve as described earlier [28], using 1 μM DAUDA as the constant concentration of labelled ligand. This displacement curve was linearized as described by Van Zoelen et al. [28], and the K_d for DAUDA was obtained from the values of slope and intercept. In addition the parameter Z_o was determined from this linearity, which equals the fraction of DAUDA which becomes bound after incubation of 1 μM DAUDA with FABP.

The K_i values of competing ligands were subsequently determined by an equivalent competition principle, by reformulating equation [10C] of reference [27] according to

$$\frac{L_i - L_s^u}{L_s^u (1 - Z_o.\Phi)} = \frac{K_i - K_d}{K_d}$$

In this equation L_i is the added concentration of competitor, L_s^u is the concentration of DAUDA required to get the same percentage of displacement as found with L_i in the mathematically converted DAUDA saturation curve, K_i the dissociation constant for the competitor and K_d the dissociation constant for DAUDA. The value of Φ is given by the fraction of fluorescence intensity, remaining after addition of competitor. The above equation assumes that DAUDA fluorescence can be fully competed by an excess concentration of competitor.

Other procedures

SDS-PAGE and Western blotting were performed as earlier described [32]. The rabbit anti-(human L-FABP) antiserum was obtained as given in [33]. Protein was determined with the Lowry procedure and corrected by amino acid analysis. Recombinant human M-FABP was

prepared as previously described [8].

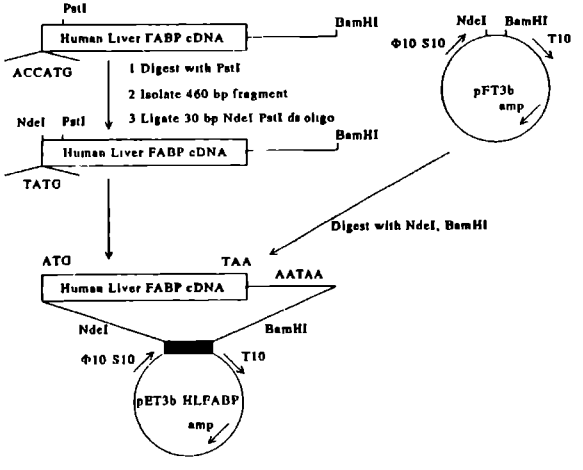

Fig. 1: **Strategy for the construction of the L-FABP expression vector pET-3b/L-FABP.** The box represents the complete coding region of L-FABP cDNA. The sequence ATG of the native cDNA was changed by digestion at the PstI site and ligation of a 30 bp synthetic oligonucleotide containing at the 3'end a PstI sticky end and at the 5'end a NdeI sticky end, as described in detail in experimental procedures. The new sequence was ligated into pET-3b to create the pET-3b/L-FABP vector. This vector has the Shine Delgarno translation initiation signal s10 between the unique NdeI site and the ϕ10 T7 promoter, a ϕT transcription terminator downstream of a BamHI site, and confers resistance to ampicillin.

RESULTS AND DISCUSSION

Synthesis and purification of recombinant L-FABP

The strategy for constructing the expression vector pET-3b/L-FABP relied on modifying the 5' end of the L-FABP cDNA with a synthetic oligonucleotide to position an NdeI site at the ATG start site. This allowed the cloning of the complete coding region of the L-FABP cDNA downstream the T7-RNA polymerase promoter (Fig. 1). *E. coli* K12 strain BL21(DE3) transformed with pET-3b/L-FABP did not show recombinant L-FABP after 3 h and overnight induction with IPTG. In vitro transcription and translation of the L-FABP cDNA and immunoprecipitation of the in vitro translation mixture with antisera raised against L-FABP revealed, however, immunoreactive protein [7]. This indicates the cDNA has no out of frame mutations, but the expression of FABP in *E. coli* is undectable. A slight leakage of the lacUV5 promoter upstream of the T7 RNA polymerase gene may cause a basal level of T7 RNA-polymerase production and target gene transcription in uninduced cells [29]. Therefore it might be that in comparison to uninduced cells no L-FABP is visible in induced cells on SDS-PAGE. To overcome this problem we used *E. coli* strain K12 BL21(DE3)/pLys S for transformation

but got similar results. To reduce the synthesis of bacterial proteins and improve L-FABP expression rifampicin was added to cultures [29]. This resulted in low amounts of recombinant L-FABP only after overnight expression (Fig. 2 and 3). The use of more rich media like TB and SBLH medium, carbenicillin instead of ampicillin, induction at higher cell density, induction with 1 and 2 mM-IPTG and growth at 30°C did not improve L-FABP expression.

The low expression might be explained by the fact that the Shine Delgarno ribosome binding site is not in optimal distance of the translational start. Studies on the expression of rat L-FABP revealed that no expression could be detected when the Shine Delgarno sequence was at 15 bp of the start codon. However within a distance of 6 bp of the start codon expression was found [16]. A second reason is that the L-FABP cDNA contains amino acid codons which are less preferential for the used bacterial strain [34]. Synthetic L-FABP cDNA prepared from oligonucleotide primers, using *E. coli* preferential codons, revealed a 20-fold higher expression than the isolated rat L-FABP cDNA [16,17].

For purification of the recombinant human L-FABP the bacterial lysate was precipitated with 50% and 70% ammonium sulphate and subsequently subjected to anion-exchange and gel filtration chromatography (Fig. 2). The yield was 1-2 mg/l culture. This is similar to the expression of rat L-FABP in *E. coli* [16] but much lower than of human M-FABP [8]. The recombinant protein was recognized by an antiserum evoked against isolated human L-FABP (Fig. 3).

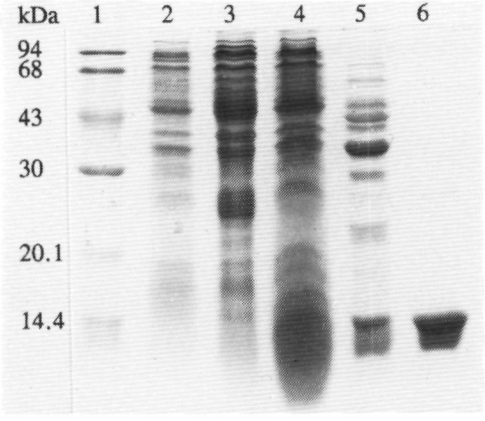

Fig. 2: Analysis of the expression and purification of recombinant L-FABP. Samples were fractionated on a 15% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, molecular mass markers; lane 2, whole cell lysate of noninduced cells (100 μg); lane 3, whole cell lysate after 3 h of induction (125 μg); lane 4, whole cell lysate after overnight induction (125 μg); lane 5, supernatant after 70% saturation with ammonium sulphate (100 μg); lane 6, pure L-FABP after anion exchange chromatography and gel filtration (15 μg).

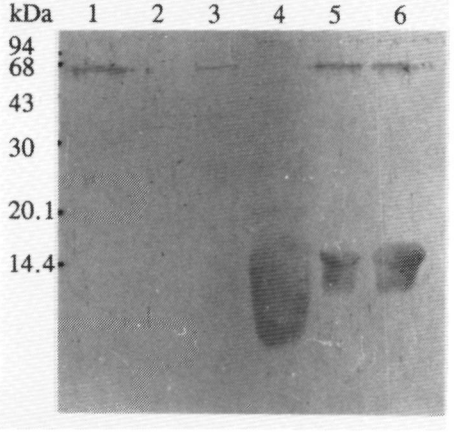

Fig. 3: Immunostained Western blot of recombinant L-FABP. Samples were fractionated on a 15% SDS-PAGE and transferred to nitrocellulose. The blot was stained with anti-(human L-FABP) antiserum. Lane 1, molecular mass markers; lane 2, whole cell lysate of non induced cells (100 μg); lane 3, whole cell lysate after 3 h of induction (125 μg); lane 4, whole cell lysate after overnight induction (125 μg); lane 5, supernatant after 70% saturation with ammonium sulphate (100 μg); lane 6, pure L-FABP after anion exchange chromatography

Analysis of the binding characteristics of recombinant L-FABP and M-FABP.

Analysis by radiochemical assay.

First the binding affinity for [1-^{14}C]oleic acid was analyzed with the Lipidex method. Scatchard plots revealed a K_d of 0.90 ± 0.04 μM and 0.43 ± 0.03 μM (means ± S.D. of 4 experiments) for human L-FABP and M-FABP, respectively. These values are in good agreement with earlier found values for isolated or recombinant proteins [8,33,35]. Delipidation was essential, since without this procedure apparent K_d values of oleic acid of 0.51 ± 0.05 μM and 0.20 ± 0.03 μM were found for human L-FABP and M-FABP, respectively.

To obtain the binding affinity for various ligands the displacement percentage of [1-^{14}C]oleic acid by these ligands was determined for both L-FABP and M-FABP. From displacement curves, obtained by addition of 1-10 μM unlabeled oleic acid to 1 μM [1-^{14}C]oleic acid and protein samples, the oleic acid concentration required to obtain the same displacement percentage as with the specific competitor, could be established. With the formula described in the experimental procedures the K_i for the specific competitor was calculated. Table 1 gives the K_i values of L-FABP and M-FABP binding to fatty acids varying in chain length and the number and place of double bonds and for various hydrophobic ligands. Although the differences in K_i values of fatty acids are relatively small data show that the recombinant L-FABP and M-FABP have both a specific preference. L-FABP shows a high affinity for all saturated and unsaturated fatty acids with a chain of 15 C atoms and longer up to $C_{24:1}$. M-FABP shows a high preference for binding unsaturated fatty acids, especially with 18 C atoms. Both proteins have similar K_i values for cis- and trans-parinaric acid. Remarkable is the difference in K_i for these ligands which suggests that a cis double bound at C-15 causes a weaker interaction. A cis double bond at C-6 in petroselenic acid shows a similar effect for both FABP types.

Comparison of our data on delipidated L-FABP with those found by others, who usually did not delipidate, shows some differences. Takikawa and Kaplowitz estimated a much higher affinity of human L-FABP for oleic acid [36]. Bass et al. found similar K_d values of stearic, linoleic and archidonic acid binding to rat L-FABP but their values for palmitic, oleic and erucic acid differ from ours [37]. Miller and Cistola [38] reported affinities of rat L-FABP to oleic acid in a similar range as we found for human L-FABP. Nemecz et al. [39] found a much lower affinity of rat L-FABP for stearic, oleic and linoleic acid, but a higher for cis-parinaric acid. Their K_d value for trans-parinaric acid does not significantly differ from our value. Lowe et al. [40] also found higher K_d values of palmitic, oleic and archidonic acid with rat L-FABP than we [33]. The displacement percentages observed for human M-FABP are in good agreement with those found by Peeters et al. [8,35].

Several reports [for refs see 5 and 26] describe the interaction of FABPs with other ligands than fatty acids. Our data (Table 2) indicate that M-FABP binds besides fatty acids only testosterone and oestradiol with high affinity. However the human L-FABP binds cellular metabolites like the palmitoyl derivatives, lysolecithin, deoxycholic acid, prostaglandins, steroid hormones, hexadecanedioic acid and eicosanol. Also retinoids, 1,25-dihydroxy vitamin D_3,

clofibric acid, bromothalein and inhibitors of carntine palmitoyltransferase (tetradecylglycidic acid and POCA) are bound by L-FABP. Their affinities are generally weaker than those of the long-chain fatty acids. Only bromothalein and prostaglandin A_1 show a higher affinity than oleic acid.Takikawa and Kaplowitz found binding of deoxycholic acid by human L-FABP with a lower affinity [36]. For palmitoyl-CoA binding to rat L-FABP, lower K_d values were found by others [37,41,42], but their FABP preparations isolated from rat liver may contain some acyl-CoA binding protein [43]. The binding affinity of prostaglandins for human liver FABP decreases in the order $PGA_1 > PGE_2$ and $PGE_1 > PGF_{2\alpha}$, prostacyclin and thromboxane B_2. These data agree with the preferential binding of growth-inhibitory prostaglandins to rat liver FABP [44]. Testosterone binds more avidly than oestradiol and ethynyloestradiol in contrast to previous observations [45]. Retinol, retinoic acid and 1,25-dihydroxy vitamin D_3 show a moderate affinity to liver FABP, but vitamin E and K_3 only a low affinity. The displacement of oleic acid by hexadecanedioic acid, but not by dodecanedioic acid is of interest in relation to the differences in their inducing activity of both FABP and peroxisomes in cultured hepatocytes [46]. In spite of the generally lower affinity for the other hydrophobic ligands than for fatty acids, L-FABP might be involved in their transport and biotransformation in liver, intestine and kidney and also protect the cell against toxicity of these ligands.

The fatty acids with a fluorescent reporter group, DAUDA and the anthroyloxy fatty acids, are bound with higher affinity by L-FABP than by M-FABP, except 12-(9-anthroyloxy)-oleic acid (Table 1). Storch et al. reported a similar K_d value of 2-(9 anthroyloxy)palmitic acid for binding to rat L-FABP [22]. The location of the fluorescent group at C-12 seems to decrease the affinity for L-FABP whereas location at the end of the fatty acid increases the affinity in comparison to the native fatty acid. L-FABP is suggested to have a more open and hydrophobic binding pocket than M-FABP [22-24] which explains the difference in K_i values of the fluorescent fatty acids between both proteins. The fluorescent reporter group rather than the fatty acid may interact with the protein which might explain the differences in affinity with the native fatty acid.

The tertiary structure of M-FABP and intestinal FABP show a large extent of similarity [15]. For L-FABP the three-dimensional structure is not known up to now. NMR studies with ^{13}C-carboxy-labeled fatty acids indicated a more solvent-accessible binding environment for L-FABP than for intestinal FABP [47]. The location of the carboxylic group near the aqueous surface of L-FABP was also suggested by the variations of quantum yields in the interaction of L-FABP with a series of fluorescent fatty acids with the anthroyl group attached at different C atoms [22]. The carbon chain is present in a nonpolar environment in a fairly constrained configuration. The constraint at the C-6/C-7 position of the acyl chain would be higher than at the methyl or carboxyl end [22]. Our results with the anthroyloxy-labeled fatty acids sustain this latter conclusion. The 12-(9-anthroyloxy)oleic acid has the lowest K_i value. The comparable fluorescence enhancement indicates, however, a similar interaction of the anthroylgroup of 2-(9-anthroyloxy)palmitic acid and 12-(9-anthroyloxy)oleic acid with L-FABP.

Table 1 Displacement of [1-^{14}C]oleic acid and binding affinity of human recombinant L-FABP and M-FABP for various fatty acids. Displacement is given as the percentage of [1-^{14}C]oleic acid binding to FABP in the absence of competitor. Concentrations were 0.2 μM L-FABP or 0.28 μM M-FABP, 1 μM [1-^{14}C]oleic acid and 1 μM competitor. The K_d for [1-^{14}C]oleic acid was determined by Scatchard analysis of binding data, the K_i for the competitors by the method of Van Zoelen [27] as described in detail in experimental procedures. Values are means ± S.D. of four independent experiments.

	L-FABP		M-FABP	
Ligand	% displacement	K_i (μM)	% displacement	K_i (μM)
Undecanoic acid (11:0)	24 ± 1	1.68 ± 0.45	19 ± 6	1.18 ± 0.41
Lauric acid (12:0)	33 ± 1	1.08 ± 0.36	19 ± 4	1.09 ± 0.14
Myristic acid (14:0)	26 ± 5	1.42 ± 0.43	15 ± 4	1.45 ± 0.26
Pentadecanoic acid (15:0)	34 ± 3	1.02 ± 0.26	20 ± 5	1.05 ± 0.21
Palmitic acid (16:0)	30 ± 3	1.23 ± 0.31	20 ± 8	1.11 ± 0.37
Stearic acid (18:0)	35 ± 2	0.97 ± 0.26	14 ± 3	1.64 ± 0.44
Nonadecanoic acid (19:0)	17 ± 3	1.24 ± 0.48	2 ± 2	> 10
Palmitoleic acid (c9, 16:1)	29 ± 1	1.34 ± 0.40	28 ± 5	0.67 ± 0.12
Petroselenic acid (c6, 18:1)	25 ± 3	1.67 ± 0.41	19 ± 8	1.36 ± 0.74
Oleic acid (c9, 18:1)	39 ± 7	0.90 ± 0.04	37 ± 1	0.43 ± 0.03
Elaidic acid (t9, 18:1)	31 ± 2	1.23 ± 0.33	48 ± 7	0.28 ± 0.05
Linoleic acid (c9,12, 18:2)	34 ± 1	1.05 ± 0.37	34 ± 6	0.51 ± 0.09
Linolenic acid (c9,12,15, 18:3)	38 ± 1	0.86 ± 0.25	35 ± 7	0.48 ± 0.10
Cis-parinaric acid (c9,15,t11,13, 18:4)	31 ± 2	1.32 ± 0.33	18 ± 1	1.26 ± 0.23
Trans-parinaric acid (t9,11,13,15, 18:4)	53 ± 3	0.46 ± 0.14	35 ± 1	0.49 ± 0.03
Arachidonic acid (c5,8,11,14, 20:4)	39 ± 3	0.84 ± 0.23	21 ± 7	0.95 ± 0.35
Erucic acid (c13, 22:1)	58 ± 1	0.39 ± 0.14	28 ± 7	0.70 ± 0.19
Cervonic acid (c4,7,10,13,16,19, 22:6)	39 ± 3	0.82 ± 0.23	15 ± 4	1.43 ± 0.28
Nervonic acid (c15, 24:1)	50 ± 1	0.55 ± 0.22	19 ± 7	1.20 ± 0.37
2-Br-palmitic acid	35 ± 1	0.95 ± 0.30	26 ± 1	0.64 ± 0.05
2-Hydroxy-palmitic acid	34 ± 1	1.03 ± 0.31	15 ± 5	1.52 ± 0.50
2-(9-Anthroyloxy) palmitic acid	63 ± 4	0.29 ± 0.07	17 ± 2	1.36 ± 0.33
12-(9-Anthroyloxy) oleic acid	14 ± 1	4 34 ± 1.03	25 ± 1	0.80 ± 0.07
16-(9-Anthroyloxy) palmitic acid	92 ± 3	< 0.05	24 ± 1	0.83 ± 0.08
11-Dansylamino undecanoic acid	73 ± 2	0.15 ± 0 08	19 ± 2	1.11 ± 0.18

Table 2 Displacement of [1-^{14}C]oleic acid and binding affinity of human recombinant L-FABP and M-FABP for various ligands. Displacement is given as the percentage of [1-^{14}C]oleic acid binding to FABP in the absence of competitor. Concentrations were 0.2 μM L-FABP or 0.28 μM M-FABP, 1 μM [1-^{14}C]oleic acid and 1 μM competitor. The K_i values were determined by analysis of binding data for the competitors by the method of Van Zoelen [27] as described in detail in experimental procedures. Values are means ± S.D. of at least three independent experiments. N.D., not determined.

	L-FABP		M-FABP	
Ligand	% displacement	K_i (μM)	% displacement	K_i (μM)
Palmitoyl-CoA	26 ± 2	1.55 ± 0.40	11 ± 2	2.03 ± 0.23
Palmitoylcarnitine	35 ± 3	0.96 ± 0.25	2 ± 2	> 10
Palmitoylglycerol	30 ± 3	1.26 ± 0.30	12 ± 6	2.09 ± 0.68
Lysolecithin	29 ± 3	1.30 ± 0.32	1 ± 1	> 10
Deoxycholic acid	20 ± 3	2.23 ± 0.57	1 ± 1	> 10
Prostaglandin E_1	21 ± 6	2.08 ± 0.75	3 ± 2	> 10
Prostaglandin E_2	27 ± 10	1.08 ± 1.05	5 ± 4	4.4 ± 1.7
Prostaglandin A_1	46 ± 11	0.67 ± 0.33	3 ± 3	> 10
Prostaglandin $F_{2\alpha}$	15 ± 4	3.01 ± 0.84	4 ± 1	> 10
Prostacyclin	10 ± 8	4.33 ± 3.27	2 ± 1	> 10
Thromboxane B_2	8 ± 7	5.17 ± 3.34	2 ± 1	> 10
Testosterone	25 ± 6	1.58 ± 0.49	30 ± 10	0.63 ± 0.30
Oestradiol	11 ± 6	6.85 ± 3.59	29 ± 6	0.63 ± 0.15
Ethynyl oestradiol	6 ± 6	7.30 ± 0.27	N.D.	N.D.
Dodecanedioic acid	3 ± 3	> 10	N.D.	N.D.
Hexadecanedioic acid	24 ± 7	2.27 ± 1.02	17 ± 5	1.24 ± 0.50
Eicosanol	29 ± 1	1.34 ± 0.40	8 ± 6	3.4 ± 1.5
Retinol	27 ± 5	1.40 ± 0.36	11 ± 9	2.7 ± 2.0
Retinoic acid	20 ± 6	2.66 ± 1.24	2 ± 2	> 10
1,25 Dihydroxy vitamin D_3	21 ± 8	1.85 ± 1.56	5 ± 5	5.3 ± 4.6
Vitamin E	6 ± 4	7.90 ± 3.50	10 ± 4	2.5 ± 1.0
Vitamin K_3	12 ± 6	4.17 ± 3.39	9 ± 5	3.2 ± 1.6
Clofibric acid	30 ± 3	1.23 ± 0.31	11 ± 6	2.6 ± 1.2
Bromothalein	49 ± 2	0.56 ± 0.17	6 ± 6	3.9 ± 1.5
Tetradecylglycidic acid	31 ± 5	1.17 ± 0.35	4 ± 4	> 10
POCA	32 ± 1	1.15 ± 0.36	11 ± 6	2.09 ± 0.68

Analysis by fluorescence assay.

The relative fluorescence enhancement was for all fluorescent fatty acids markedly higher with L-FABP than with M-FABP (Table 3). There is as expected no correlation of the K_i values of the fluorescent fatty acids (Table 1) and the fluorescence enhancement data (Table 3) for both L-FABP and M-FABP. Although the K_i values of the parinaric acids are similar for both FABPs, the fluorescence increase is much higher with L-FABP. The interaction with the anthroyloxy-labeled fatty acids depends on the location of the fluorescent reporter group e.g. the 16-AP shows the strongest affinity but the lowest fluorescence increase with L-FABP. These differences relate to the different protein-ligand interactions studied in the two assays. The radiochemical displacement assay is based on the interference with the binding of the oleic acid carbon chain, whereas the fluorescence enhancement assay indicates a hydrophobic interaction of the fluorescent reporter group with the protein. The latter is clear from the interaction of rat L-FABP with the fluorescent trinitrophenyladenosine-triphosphate, but not with ATP itself [25]. The more hydrophobic binding pocket of L-FABP than of M-FABP might explain the difference in fluorescence increase of both proteins as suggested [22-24].

Table 3 Fluorescence increase of different fatty acids in reaction with human L-FABP and M-FABP. Experiments were performed with 1.0 μM fatty acid in the absence or presence of 2.0 μM L-FABP or 2.8 μM M-FABP. Optimal excitation wavelength λ_{ex} is given in nm. The fluorescence increase is calculated as the ratio of fluorescence with and without protein. Fluorescence values of DAUDA are relative to the emission without protein at 545 nm (excitation at 328 nm), since at 505 nm (excitation 345 nm) no fluorescence without protein is observed. Maximum fluorescence of the other ligands was obtained by scanning of emission between 380 and 550 nm. Values are means ± S.D. of three independent experiments.

Ligand	λex	L-FABP	M-FABP
DAUDA	345	18.3 ± 0.2	3.4 ± 0.2
2-(9-anthroyloxy)palmitic acid	365	20.0 ± 0.2	13.3 ± 0.2
12-(9-anthroyloxy)oleic acid	365	21.8 ± 0.9	5.8 ± 0.4
16-(9-anthroyloxy)palmitic acid	365	8.2 ± 0.6	2.5 ± 0.6
Cis-parinaric acid	306	15.3 ± 0.7	5.7 ± 0.4
Trans-parinaric acid	306	22.0 ± 0.4	5.5 ± 0.5

The low fluorescence enhancement does not allow displacement studies and determination of the K_i value of the fluorescent fatty acids with M-FABP. In our experiments with fluorescent probes we noticed that DAUDA is most stable and most suitable for displacement studies. First the optimal L-FABP concentration was determined to get a fluorescence signal which is sensitive enough to study displacement and at which the fatty acid concentration is saturating. With 0.2 μM L-FABP the displacement percentage with oleic acid is identical with that at lower FABP concentrations and the signal is most optimal (Fig. 4).

The binding affinity of recombinant human L-FABP for DAUDA was determined by analysis of its binding and displacement curves [28]. The K_d was established at 0.17 ± 0.05 μM (mean ± S.D. of four ex-periments) which is in excellent accordance with the K_i determined from the displacement of [1-^{14}C]oleic acid (Table 1) and the reported K_d values for binding of DAUDA to isolated rat and porcine L-FABP [17,28]. This K_d value is used in the calculation of

the K_i values of competing ligands (Table 4).

The same fatty acids and other hydrophobic ligands as used in radiochemical analysis were applied in DAUDA displacement studies with L-FABP. The displacement percentages are given for all ligands in Table 4. The preference for long-chain fatty acids is similar as in the oleic acid displacement studies. A peculiar finding was the result with petroselenic acid which does not displace DAUDA although from oleic acid displacement it appeared that this acid is bound by L-FABP. Displacement studies with DAUDA on isolated human and rat L-FABP showed a similar binding preference although the values differ a little [21,33]. Other ligands as palmitoyl-CoA, lysolecithin, bromothalein and tetradecylglycidic acid also inhibit the binding of DAUDA to L-FABP (Table 4) as previously found for isolated human and rat L-FABP [21,33]. However, palmitoylglycerol, deoxycholic acid, POCA, clofibric acid, eicosanol, prostaglandin E_1 and E_2 and retinol did not inhibit DAUDA fluorescence with human L-FABP (data not shown) in contrast to their interference with oleic acid binding.

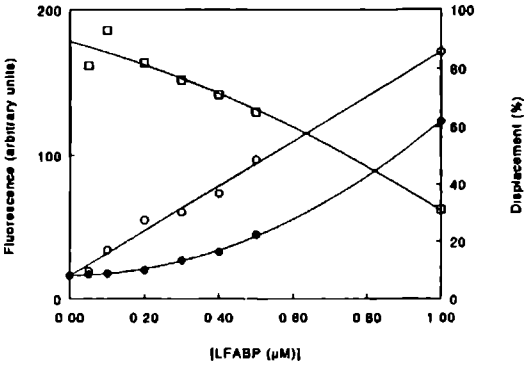

Fig. 4 Analysis of the optimal L-FABP concentration for fluorescence displacement studies with DAUDA. The fluorescence of 1 μM DAUDA with (●) or without (○) 1 μM oleic acid is given for the range of 0-1 μM L-FABP. The displacement percentage (□) is also given.

Comparison of the data of Table 4 with the radiochemical studies (Table 1) shows that the displacement percentages of DAUDA by various fatty acids are markedly higher than of oleic acid although the K_d value of DAUDA is considerably lower. The K_d value for [1-^{14}C]oleic acid binding was 0.90 μM but displacement of DAUDA gave a K_i value of 0.19 μM. The apparent K_i values of the competing ligands calculated from their displacement percentage and the DAUDA displacement curves (Table 4) appear also to differ from the K_i values obtained with [1-^{14}C]oleic acid displacement. The long-chain saturated and unsaturated fatty acids and palmitoyl-CoA, and tetradecylglycidic acid show generally a lower K_i, the other ligands a markedly higher K_i value in the DAUDA displacement studies (Table 4, and data not shown). These distinct differences between the results of both displacement assays reflect the different kind and location of interaction of the acyl carbon chain and the fluorescent reporter group with the L-FABP. The decrease of bound radioactivity is based on the competitive interaction with the binding of the acyl chain of oleic acid to L-FABP, whereas the decrease of the fluorescence signal gives only information on the competition with the fluorescent reporter group of DAUDA in its interaction with the protein.

In conclusion, we obtained only good expression of human L-FABP in *E.coli* after overnight incubation with IPTG and rifampicin. We showed that with the oleic acid displacement method the K_i values of L-FABP and M-FABP for a large series of fatty acids and other

ligands can be determined. Our results indicate that binding assays with fatty acids with fluorescent groups do not well reflect the interaction of the acyl carbon chain with the FABP. The K_d value for DAUDA was found to be similar in assays based on [1-^{14}C]oleic acid displacement and DAUDA saturation. The type-specific ligand preference of L-FABP and M-FABP might relate to the presence of both FABP types in one organ, like the kidney [6,7].

Table 4 Displacement of DAUDA and binding affinity of human recombinant L-FABP for various hydrophobic ligands. Recombinant human L-FABP (0.2 μM) was incubated with 1 μM DAUDA with or without 1 μM competitor. Fluorescence spectra were measured between 475 and 550 nm at an excitation wavelength of 345 nm. The fall in fluorescence due to the displacement of DAUDA from L-FABP by competing ligand is expressed as a percentage of the initial protein-bound DAUDA fluorescence. The K_i values of the competing ligands were calculated from their displacement percentage and the DAUDA displacement curve. All values are means ± S.D. for at least four independent experiments.

Ligand	% displacement	K_i (μM)
Undecanoic acid (11:0)	1 ± 3	> 10
Lauric acid (12:0)	1 ± 2	> 10
Myristic acid (14:0)	21 ± 4	2.94 ± 0.55
Pentadecanoic acid (15:0)	53 ± 3	0.70 ± 0.06
Palmitic acid (16:0)	73 ± 5	0 26 ± 0 07
Stearic acid (18:0)	76 ± 5	0.21 ± 0.04
Nonadecanoic acid (19:0)	71 ± 2	0.32 ± 0.05
Palmitoleic acid (c9, 16:1)	61 ± 2	0.52 ± 0.04
Petroselenic acid (c6, 18:1)	1 ± 1	> 10
Oleic acid (c9, 18:1)	78 ± 5	0.19 ± 0.04
Elaidic acid (t9, 18:1)	80 ± 3	0.17 ± 0.02
Linoleic acid (c9,12, 18:2)	70 ± 5	0.32 ± 0.08
Linolenic acid (c9,12,15, 18:3)	60 ± 2	0.54 ± 0.03
Arachidonic acid (c5,8,11,14, 20:4)	52 ± 4	0.70 ± 0.08
Erucic acid (c13, 22:1)	84 ± 3	0.15 ± 0.05
Cervonic acid (c4,7,10,13,16,19, 22.6)	41 ± 3	1.28 ± 0 20
Nervonic acid (c15, 24:1)	84 ± 1	0.15 ± 0.01
2-Br-palmitic acid	78 ± 5	0.19 ± 0.04
2-Hydroxy-palmitic acid	39 ± 4	1.48 ± 0.30
Palmitoyl-CoA	50 ± 2	0.77 ± 0.05
Palmitoylcarnitine	9 ± 7	6.40 ± 2.70
Lysolecithine	25 ± 6	2.44 ± 0.58
Bromothalein	38 ± 6	1 59 ± 0.49
Tetradecylglycidic acid	66 ± 2	0.39 ± 0.03

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CHAPTER 6

PRIMARY STRUCTURE AND BINDING CHARACTERISTICS OF LOCUST AND HUMAN MUSCLE FATTY ACID-BINDING PROTEIN

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SUMMARY

The conservation between M-FABP of *Locusta migratoria* (Lm) flight muscle and of human skeletal muscle was investigated. The locust M-FABP cDNA (632 bp) was isolated by 5' and 3' rapid amplification of cDNA ends. The identity of the locust and human M-FABP on the cDNA and the protein level is 54% and 42%, respectively. The predicted amino acid sequence of locust M-FABP indicates a molecular mass of 14,935 Da and an isoelectric point of 6.1. The locust M-FABP was expressed in *Escherichia coli*, purified by $(NH_4)_2SO_4$ precipitation, anion-exchange and gel filtration chromatography and compared with the recombinant human M-FABP with respect to immunological and binding properties. In spite of the high sequence similarity, the proteins did not show immunological cross-reactivity. The binding parameters of locust M-FABP were analyzed with radiolabeled oleic acid by the Lipidex assay and titration microcalorimetry. Both methods revealed a K_d for oleic acid of 0.5 μM and a binding stochiometry of 1 mol fatty acid/mol FABP. The ΔH, ΔG and ΔS for oleic acid binding are -146 and -36 kJ/mol and -369 J/mol K, respectively. All information obtained from binding, fluorescence and displacement studies indicated that locust M-FABP has binding characteristics similar to human M-FABP. Finally the recombinant locust M-FABP was crystallized with and without oleic acid. All crystals are trigonal in the $P3_121$ space group. The unit cell dimensions are $a=b=5.89$ nm and $c=14.42$ nm.

INTRODUCTION

Fatty acids are important molecules for energy generation and synthesis of membrane lipids. Moreover, they may function as lipid mediators and influence various cellular processes by their interaction with enzymes, membranes, ion channels, receptors or genes. In the cytoplasm of tissues the flux of fatty acids between plasma membrane and organelles, where fatty acids are converted, seems to be controled by a group of low-molecular mass proteins, the fatty acid-binding proteins (FABPs). Until now, seven vertebrate FABPs, with a amino acid sequence similarity of 25-65%, have been identified: liver, intestinal, heart or muscle, adipocyte, myelin, ileal and epidermal FABP types [1-5].

Previously we isolated human M-FABP, a protein of a 15 kDa molecular mass, a isoelectric point of 5.2 and a high immunological crossreactivity with human heart FABP [6]. Analysis of a full-length human M-FABP cDNA clone and amino acid sequencing of human M-FABP proved that muscle and heart FABPs are identical [7]. The human M-FABP has a more general tissue distribution than all other FABP types [1-5]. The recombinant human M-FABP showed preference for binding of palmitic and C_{18}-C_{22} unsaturated fatty acids [8]. The recombinant protein was crystallized and its three-dimensional structure solved [9]. The structure of human M-FABP was found to be similar to that of other members of the FABP family; containing ten anti-parallel ß-strands arranged in two orthogonal ß-sheets and two short α-helices located between the first and second ß-strand [9]. In spite of their high conformational similarity, intestinal, adipocyte, myelin and muscle FABP types bind their fatty acid differently. The muscle and myelin FABP types bind the fatty acid in an U-shape [9,10] whereas the

intestinal and adipocyte FABP types bind the fatty acid in an more extended conformation [11-14]. Amino acids involved with fatty acid binding are also different between muscle and intestinal FABP [15]. Additionally, evidence has been obtained that adipocyte and muscle FABPs contain a tyrosine phosphorylation recognition sequence [16-18].

Recently FABPs have been isolated from the invertebrate species *Schistosoma mansoni* [19], *Schistocerca gregaria* [20] and *Locusta migratoria* [21,22]. One FABP preparation obtained from midgut of *Manduca sexta* [23] was used to determine the three-dimensional overall structure. The overall structure of the protein is similar to that of the mammalian intestinal, myelin and adipocyte FABP types [24]. Locust flight muscle uses exclusively fatty acids as energy source for sustained flight activity and it is likely that FABP is involved in the intracellular fatty acid transport [21]. Immunochemical studies revealed that in the desert locust *S. gregaria* this protein is only present in adult flight muscle and comprises 18% of the total cytosolic protein [20,25]. The amino acid sequence of flight muscle FABP from *S. gregaria* seems to be highly conserved during evolution and the highest similarity of amino acid sequence (46%) was found with human M-FABP [26].

As an extension of our isolation of flight muscle FABP from *Locusta migratoria* [21,22] we determined its complete cDNA sequence and expressed the protein in *E. coli*. Since locust and human M-FABP showed a high primary structure identity we studied subsequently the immunological and binding properties of both proteins to obtain more information about a similarity of their tertiary structure. Finally we crystallized the recombinant locust M-FABP in order to begin the three-dimensional structure analysis.

MATERIALS AND METHODS

Materials

Oligonucleotide primers and the FPLC Hiload Q Sepharose column were obtained from Pharmacia Biotechnologies Int., Uppsala, Sweden; RNasin and terminal deoxynucleotidyl transferase from Promega Corporation, Madison, WI, USA; recombinant Taq DNA polymerase Amplitaq from Perkin Elmer Cetus, Norwalk, CT, USA; Moloney Murine Leukemia virus [MMuLV] RNase H^- reverse transcriptase and isopropyl ß-D-thiogalactopyranoside from Life Technologies Inc., Gaithersburg, MD, U.S.A.; Geneclean from BIO101 Inc., La Jolla, CA, U.S.A.; Klenow DNA polymerase, T4 polynucleotide kinase; proteinase K and calf alkaline intestinal phosphatase from Boehringer Mannheim, Germany; Sequenase version 2.0 from United States Biochemical, Cleveland, OH, USA; Lipidex 1000 from Canberra-Packard, Groningen, The Netherlands; 11-dansylamino-undecanoic acid, 2-(9-anthroyloxy)palmitic acid, 12-(9-anthroyloxy)oleic acid and 16-(9-anthroyloxy)palmitic acid from Molecular Probes, Junction City, OR, U.S.A.; polyethylene glycol 4000 from J.T. Baker Chem. Co., Phillipsburg, N.J., U.S.A.; [9,10-^{3}H]oleic acid [2-10 Ci/mmol], [1-^{14}C]oleic acid [55 mCi/mmol] and α-[^{35}S]thio-dATP [1000 Ci/mmol], from Amersham International, Little Chalford, U.K.. All other reagents were of analytical grade.

Methods

Isolation and cloning of a full-length cDNA of locust M-FABP

Locusta migratoria was reared in the laboratory under crowded conditions as described [27]. Locust flight muscle FABP was isolated as described by Van der Horst et al.[22]. The N-terminal amino acid sequence of purified locust FABP was determined by automated Edman degradation. The amino acid sequence of the first 41 residues is as follows H_2N-Val-Lys-Glu-Phe-Ala-Gly-Ile-Lys-Tyr-Lys-Leu-Asp-Ser-Gln-Thr-Asn-Phe-Glu-Glu-Tyr-Met-Lys-Ala-Ile-Gly-Val-Gly-Ala-Ile-Glu-Arg-Lys-Ala-Gly-Leu-Ala-Leu-Ser-Pro-Val-Ile [22].

Total RNA was extracted from 6.4 g flight muscle by the procedure of Auffray et al. and Puissant et al. [28,29]. Poly(A)-rich RNA was selected on oligo(dT)-cellulose [30]. Northern blot analysis was carried out as previously described [31]. The principles of 5' and 3' RACE procedures are given in refs 32 and 33. The sequence of the primers used is given in Fig. 1.

First strand synthesis of 3' RACE was carried out with 20 μg total RNA in a total volume of 20 μl containing 50 mM Tris/HCl (pH 8.3)/75 mM KCl/3 mM $MgCl_2$/125 μM dNTPs/40 units RNasin/0.5 μg oligo-dT primer and 400 units M-MuLV reverse transcriptase. The reverse oligo-dT primer was added to the RNA sample, the mixture was heated for 10 min at 70°C and quenched on ice. The reverse transcription reaction was carried out for 1 h at 37°C and for 30 min at 45°C.

The PCR amplification reactions were carried out in a total volume of 100 μl containing 2 μl of the reverse transcription reaction mixture, 0.5 μg reverse oligo-dT and 0.5 μg forward degenerate primer, 10 mM Tris/HCl (pH 8.3)/50 mM KCl/1.5 mM $MgCl_2$/0.001 % gelatin/250 μM-dNTPs and 2.5 units Amplitaq. After 8 min denaturation at 94°C, 40 cycles of amplification were carried out by using a step program (94°C, 1 min; 37°C, 1.5 min; 72°C, 2 min) followed by a 2 min final extension at 72°C.

First strand synthesis of 5' RACE with reverse primer 1 was carried out as described above using instead of 20 μg total RNA, 10 μg poly(A)-rich RNA. The RNA was hydrolyzed by adding an equal volume of 0.3 M NaOH/30 mM EDTA and boiling for 5 min. The first strand of cDNA was purified with Geneclean. A quarter of the purified first strand was used directly for dG-tailing. The first strand cDNA was denatured at 70°C for 5 min, chilled on ice, and the remainder of the reaction components was added. The final composition was 10 mM Tris/HCl (pH 8.4), 25 mM KCl, 1.25 mM $MgCl_2$, 50 μg/ml bovine serum albumin, 0.2 mM dGTP and 10 units terminal deoxynucleotidyl transferase. Reaction mixtures were incubated for 10 min at 37°C, then for 10 min at 65°C. Tailed cDNA was purified with Geneclean and ¼ of the volume was used for amplification during PCR with an anchor and a nested reverse primer 2. PCR reaction buffer was the same as described above, but the cycling conditions were 94°C 1 min; 55°C 1 min; 72°C 2 min.

The PCR products were subjected to electrophoresis on 1.5% agarose gel and bands of expected length were cut out. The agarose was removed by the phenol freeze fracture method

[34]. The DNA was subsequently treated with proteinase K to remove Taq polymerase contamination, with Klenow DNA polymerase to fill in the protruding ends and with T4 polynucleotide kinase to phosphorylate the 5' terminus of the cDNA ends [35,36]. The cDNA fragments were initially ligated to each other and subsequently digested with NcoI and BamHI. The digestion product was ligated with a modified PGEM5 Zf(+) vector, which had previously been digested with the same enzymes. Nucleotide sequencing was performed using the dideoxy chain termination technique using [^{35}S]thio-dATP and the Sequenase kit either on double stranded or single stranded PGEM vectors. The nucleotide sequence from each strand was determined to confirm the data.

Expression of locust M-FABP in *Escherichia coli* and purification

The primers used during 3' RACE amplification by PCR contain recognition sites for the restriction enzymes NcoI and BamHI, which facilitate cloning in pET3d. The sequenced locust M-FABP cDNA was isolated after digestion with NcoI and BamHI and ligated with pET3d vector previously digested with the same enzymes.

The plasmid pET3d/locust M-FABP was used to transform the E.coli K12 strain BL21-(DE3) [37]. The transformed cells were plated onto agar plates constituted with 2x TY medium containing 100 μg/ml ampicillin. An overnight culture of 50 ml 2x TY/ampicillin medium was made from a freshly streaked plate and 1 ml was used to inoculate 80 ml 2x TY/ampicillin medium. This was incubated at 37°C until cell density (A_{600}) was 0.5. From this culture 5 ml were used to inoculate 12 x 1.5 l of 2x TY/ampicillin. When A_{600} was between 0.7-0.9, isopropyl ß-D-thiogalactopyranoside at a final concentration of 0.5 mM was added to induce the synthesis of T7 RNA polymerase. The cultures were grown for 4 h at 37°C.

Purification of recombinant locust M-FABP

Induced cells were harvested by centrifugation at 5000 x g for 20 min. The pellets were washed with 150 mM NaCl/5.4 mM Na-phosphate/1.3 mM K-phosphate (pH 7.4), frozen at -80 °C to facilitate cell lysis and resuspended in 50 mM Tris/HCl (pH 7.5). The resuspended pellets were freeze-thawed 3 times and sonified 3 times for 30 sec with the broad tip of a Branson sonifier. The suspension was centrifuged for 20 min at 30,000 x g, the supernatant was saturated to 50% with ammonium sulphate and stirred for 1 h at 4°C. After centrifugation for 20 min at 30,000 x g the supernatant was dialyzed for 48 h against 10 mM Tris/HCl (pH 7.5). After batchwise delipidation with Lipidex 1000 the dialysate was passed through a Q Sepharose HR 26/10 FPLC column. The M-FABP was eluted with 50 mM Tris/HCl (pH 8.0). Column fractions were analyzed by SDS-PAGE and Western blotting. Pure locust M-FABP fractions were pooled. Gel filtration on Sephacryl S-100 (100x3 cm) was applied to obtain M-FABP from impure fractions.

Characterization of recombinant locust M-FABP

Titration calorimetry

Titration calorimetry was performed using a MicroCal OMEGA differential titration calorimeter (MicroCal Inc., Northampton, MA, U.S.A.) [38]. Twenty five 4-μl-aliquots of delipidated locust M-FABP sample (0.55 mM) were injected into 1.4 ml 15-25 μM [9,10-^{3}H]oleic acid solution in 20 mM Tris/HCl (pH 8.0). All titrations were executed at 25°C. The fatty acid concentration was determined after the experiment by analysis of the radioactivity. The data were processed using the software package ORIGIN (provided with the MicroCal instrument). A function that modelled one class of independent binding sites was used to fit the data [38]. The association and dissociation constants (K_a and K_d), molar binding stoichiometry and enthalpy change (ΔH) were determined from the fitted curve. The changes of Gibbs free energy and entropy were calculated using the equations $\Delta G = -RT\ln K_a$ and $\Delta S = (\Delta H - \Delta G)/T$, respectively, where R is the gas constant and T is the absolute temperature in K.

Fatty acid-binding assays

Immediately before binding assays, FABP preparations were delipidated with the Lipidex procedure [39]. All binding assays were carried out in a volume of 0.5 ml 10 mM Tris/HCl (pH 8.0)/ 1% ethanol at 37 °C. K_d values for the binding of recombinant locust and human M-FABP to [1-^{14}C]oleic acid were determined with the Lipidex procedure and Scatchard analysis [39]. The K_i values for binding of other fatty acids were determined by displacement analysis according to the method of van Zoelen [40]. Displacement curves were made with the Lipidex procedure by addition of 1 μM [1-^{14}C]oleic acid in the presence of unlabeled oleic acid (0-10 μM) to 0.4 μM locust M-FABP or 0.28 μM human M-FABP. Subsequently the displacement percentage of [1-^{14}C]oleic acid by various hydrophobic ligands was analyzed by addition of 1 μM [1-^{14}C]oleic acid and 1 μM competitor to the same amount of FABP. From the displacement curve the concentration of unlabeled oleic acid was read which is needed to obtain the same percentage of displacement as with 1 μM competitor. Van Zoelen [40] showed that the K_i value of the competitor can be calculated with the formula:

$$\frac{L_i - L_s^u}{L_s^u\,(1\text{-}cpm^{bound}/cpm^{added})} = \frac{K_i - K_d}{K_d}$$

In this equation L_i is the added concentration of competitor, L_s^u is the concentration of non-labeled oleic acid required to obtain the same percentage of displacement as found with L_i, K_i the dissociation constant for the competitor and K_d the dissociation constant for oleic acid. The correction term ($1 - cpm^{added}/cpm^{bound}$) is included to account for the fact that the free ligand concentration can be significantly lower than the ligand concentration added.

Fluorescence enhancement of various fluorescent fatty acids with recombinant locust and human M-FABP.

All fluorescence experiments were carried out at a Shimadzu-500 fluorometer. The fluorescence was analyzed for 1.0 μM fluorescent fatty acid in the absence or presence of 4.0 μM locust M-FABP or 2.8 μM human M-FABP in 1 ml 10 mM Tris/HCl (pH 8.0)/1% ethanol at 25 °C. Excitation and emission wavelengths are given in the table.

Other procedures

Standard procedures were used for SDS-PAGE and Western blotting [41]. In the latter procedure we used AMPPD (Tropix) instead of 4-chloro-1-naphtol. The antisera used were rabbit polyclonal antisera against recombinant human M-FABP and liver FABP and a monoclonal and polyclonal antiserum against locust M-FABP. Recombinant human M-FABP was prepared as previously described [8]. Protein was determined by the Lowry procedure and corrected on the basis of amino acid analysis.

Crystallization and X-ray diffraction studies on recombinant locust M-FABP

Crystals were obtained using the hanging drop vapor diffusion method [42]. Four μl of protein solution 40 mg/ml in 100 mM PIPES buffer (pH 7.1), 0.05% sodium azide were mixed with 4 μl of a solution of 30% polyethylene glycol 4000 in the same buffer (precipitant solution). The resulting 8 μl drop was placed on a silanized coverslip, inverted and sealed over a well containing 1 ml of the precipitant solution. The first crystals appeared after three days at a temperature of 20°C. After seven days the maximum dimensions of the crystals were 0.4 x 0.3 x 0.2 mm.

A crystal mounted in a quartz capillary was subjected to X-ray diffraction. The data were collected on a Siemens Area Detector system coupled to a Rigaku RU-200 rotating anode X-ray generator, operating at 50 kV and 85 mA. A copper anode was used to produce X-rays and a graphite monochromator was employed to select the Cu-Kα radiation (λ=0.1542 nm). A 0.3 mm collimator was chosen and the crystal-to-detector distance was set at 14 cm. Four runs, one phi scan and three omega scans, were performed with a frame width of 0.25 degrees and an exposure time of 90 s per frame.

The diffraction data were analyzed using the XENGEN V.2.0 programs package (Siemens Analytical X-rays Instruments Inc., Madison, WI, U.S.A.) running on a Silicon Graphics IRIS workstation. The 720 frames processed resulted in the integration of 23,729 observed reflections. The program PRECESS contained in the PHASES package was used to visualize pseudo-precession pictures of the unreduced diffraction in order to determine the space group.

RESULTS AND DISCUSSION

Isolation of a full length cDNA of locust flight muscle FABP

The amino acid sequence of locust flight muscle FABP, isolated from adult male insects, was determined by automated Edman degradation. The N-terminal sequence was not blocked in contrast to most FABPs purified from tissue extracts. Total RNA was reverse transcribed with an oligo-$(dT)_{17}$ adaptor primer and subsequently this primer and a degenerate primer deduced from the first 10 N-terminal amino acids of locust M-FABP was used in a 3' RACE (Fig. 1 upper panel). The cloned PCR fragment revealed the complete coding region and the 3' untranslated region. Northern blot analysis, using this partial cDNA as a probe, indicated that the transcript was approximately 0.6 kb in length and that the 5' untranslated region will be short. The 5' untranslated region and the coding region encoded by the degenerate primer was isolated by 5' RACE. After first strand synthesis with primer 1, hydrolysis of the RNA and purification of the first strand, a dG-tail was attached. After a second purification the 5' RACE PCR was applied with an oligo-(dC) adaptor primer and the nested primer 2 (Fig. 1 lower panel). The PCR product encoded a 54 bp 5' untranslated region and nearly the complete coding region of the cDNA. The 5' and 3' RACE as described above resulted in the isolation of a full length cDNA (632 bp) encoding the locust M-FABP (Fig. 2). The start codon is located on position 67. The stop codon and polyadenylation signal are at position 469 and 603, respectively. The sequence predicts a protein of 133 amino acids with a calculated molecular mass of 14,935 Da and isoelectric point of 6.13.

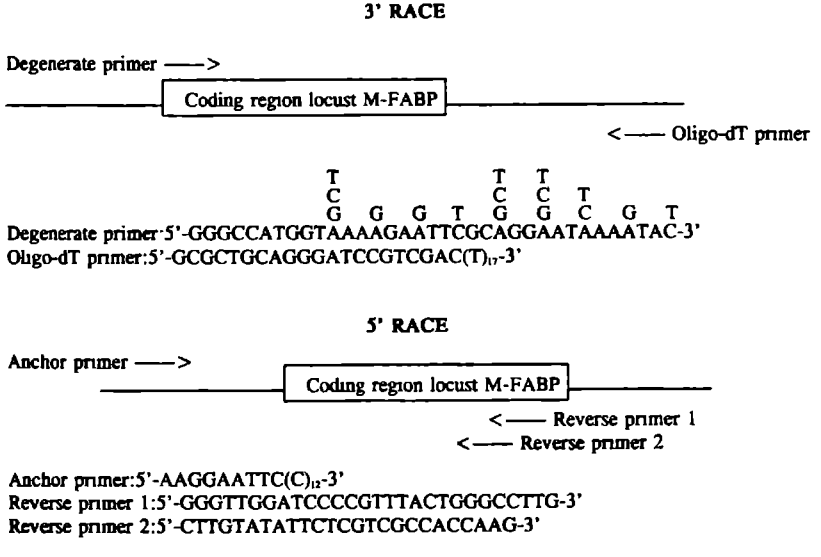

Fig. 1 Procedure of cDNA isolation. The procedure of isolation of the full-length cDNA of locust M-FABP and the primers used are given.

```
      GGGGGGGGGGGGGACTTCTCAGCCAGTTCCTGTCTACTGCACTGCCACAGCCGCACAGCCGCCGCC
      1

ATG GTC AAG GAG TTC GCC GGC ATC AAG TAC AAG CTC GAC TCG CAG ACC AAC TTC
Met Val Lys Glu Phe Ala Gly Ile Lys Tyr Lys Leu Asp Ser Gln Thr Asn Phe

GAG GAG TAC ATG AAG GCC ATC GGT GTG GGC GCC ATT GAG CGA AAG GCT GGT CTG
Glu Glu Tyr Met Lys Ala Ile Gly Val Gly Ala Ile Glu Arg Lys Ala Gly Leu

GCG CTG TCC CCG GTG ATC GAA CTG GAG GTC CTG GAC GGT GAC AAG TTC AAG CTC
Ala Leu Ser Pro Val Ile Glu Leu Glu Val Leu Asp Gly Asp Lys Phe Lys Leu

ACC TCC AAG ACC GCC ATC AAG AAC ACC GAG TTC ACC TTC AAG CTG GGC GAG GAG
Thr Ser Lys Thr Ala Ile Lys Asn Thr Glu Phe Thr Phe Lys Leu Gly Glu Glu

TTC GAC GAG GAC ACC TTG GAC GGC CGC AAG GTC AAG TCT ATC ATC ACC CAG GAC
Phe Asp Glu Asp Thr Leu Asp Gly Arg Lys Val Lys Ser Ile Ile Thr Gln Asp

GGG CCC AAC AAG CTG GTC CAC GAG CAG AAG GGT GAC CAC CCC ACT ATC ATC ATC
Gly Pro Asn Lys Leu Val His Glu Gln Lys Gly Asp His Pro Thr Ile Ile Ile

CGC GAG TTC TCC AAG GAG CAG TGC GTC ATC ACG ATT AAA CTG GGC GAC TTG GTG
Arg Glu Phe Ser Lys Glu Gln Cys Val Ile Thr Ile Lys Leu Gly Asp Leu Val


GCG ACG AGA ATA TAC AAG GCC CAG TGAACGGGCAACCAACCCACCGACTTTCTGTTGTTTTCA
Ala Thr Arg Ile Tyr Lys Ala Gln End

GCATTAAACGTAATTTTCAGTTTACTGTGTGATCAGAAGCTTTCGTTTCCTTTTTTAAGTAATATGAGCTT

GGTGTGTTTCTAAATGAATCATTTTTTACAATTTCACAAAAAAAAAAAAAAAAA
                                                        632
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Fig. 2 Full-length cDNA sequence of locust flight muscle FABP. The position of the start (67), termination (469) and polyadenylation (603) are shaded.

omparison of cDNA and amino acid sequence of locust M-FABP with other FABPs

In order to make a comparison between the primary structure of the locust M-FABP and ther M-FABPs, we aligned both the cDNA and amino acid sequences (Table 1, Fig.3). The artially elucidated cDNA sequence encoding the M-FABP of *Schistocerca gregaria* [26] is the ost similar to that of locust M-FABP with 92% identity. The identity displayed is higher, 3%, if the two amino acid sequences are compared. The identity of the human M-FABP [7]

Table 1 The identity/similarity between invertebrate FABPs and human muscle FABP
The percentage of identity/similarity is given on amino acid and cDNA level for M-FABPs from *Locusta migratoria* (Lm), *Schistocerca gregaria* (Sg) [26], *Schistosoma mansoni* (Sm) [19] and *Homo sapiens* (Hs) [7] and for midgut FABPs from *Manduca sexta* (Ms) [23].
The percentage identity with Sg M-FABP cDNA could not be determined (nd) since only a part of it was isolated and sequenced [26]. For the elucidated part the identity with Lm M-FABP cDNA is 92%.

Percent identity/similarity for amino acid alignment

	Lm M-FABP	Sg M-FABP	Sm M-FABP	Hs M-FABP	Ms FABP1	Ms FABP2
Lm M-FABP	-	97.7/97.7	36.3/38.2	41.9/46.4	26.5/30.9	32.6/34.1
Sg M-FABP	nd	-	36.3/38.2	41.2/45.7	26.5/30.9	32.6/34.1
Sm M-FABP	48.3	nd	-	43.0/44.5	23.5/26.5	24.8/30.4
Hs M-FABP	54.3	nd	50.7	-	26.6/29.2	32.6/35.0
Ms FABP1	48.9	nd	45.0	43.0	-	55.7/56.8
Ms FABP2	49.3	nd	45.4	48.1	61.4	-
	Lm M-FABP	Sg M-FABP	Sm M-FABP	Hs M-FABP	Ms FABP1	Ms FABP2

Percent identity for cDNA alignment

```
Lm-M-FABP -VKEFAGIKYKLDSQTNFEEYMKAIGVGAIERKAGLALSPVIELEVLDGDKFKLTSKTAI 58
Sg-M-FABP -VKEFAGIKYKLDSQTNFEEYMKAIGVGAIERKAGLALSPVIELEILDGDKFKLTSKTAI 58
Sm-M-FABP M-SSFLG-KWKLSESHNFDAVMSKLGVSWATRQIGNTVTPTVTFT-MDGDKMTMLTESTF 55
Hs-M-FABP MVDAFLG-TWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIE-KNGDILTLKTHSTF 56
              * *   **     **.  *  .**.   *. .    *   .   .**    . . ...

Lm-M-FABP KNTEFTFKLGEEFDEDTLDGRKVKSIITQDGPNKLVHEQK-GDHPTIIIREFSKEQCVIT 117
Sg-M-FABP KNTEFTFKLGEEFDEETLDGRKVKSTITQDGPNKLVHEQK-GDHPTIIIREFSKEQCVIT 117
Sm-M-FABP KNLSCTFKFGEEFDEKTSDGRNVKSVVEKNSESKLTQTQVDPKNTTVIVREVDGDTMKTT 115
Hs-M-FABP KNTEISFKLGVEFDETTADDRKVKSIVTLDG-GKLVHLQKWDGQETTLVRELIDGKLILT 115
          **   .**.* **** * *.*.*** .  ..  ** . +    . * ..**        *

Lm-M-FABP IKLGDLVATRIY-KAQ  132
Sg-M-FABP IKLGDLVATRIY-KAQ  132
Sm-M-FABP VTVGDVTAIRNYKRLS  131
Hs-M-FABP LTHGTAVCTRTYEKEA  131
          .  *     * * .
```

Fig. 3 Alignment of M-FABPs from three invertebrates and human. The amino acid sequence of *Locusta migratoria* (Lm), *Schistocerca gregaria* (Sg) [26], *Schistosoma mansoni* (Sm) [19] and *Homo sapiens* (Hs) [7] M-FABP are compared on similar residues. Amino acids which are identical or are conservatively substituted are marked by asterisks or points, respectively.

Table 2 Amino acid side chains of human M-FABP within 0.45 nm from the fatty acid carbon atoms and their counterpart in locust M-FABP.

Carbon atom	Amino acid M-FABP	
fatty acid	Human	Locust
C-2	Phe16, Arg126	Phe16, Arg126
C-3	-	-
C-4	Phe16	Phe16
C-5	-	-
C-6	Asp76	Asp76
C-7	Asp76	Asp76
C-8	Ala75, Asp76	Leu77, Asp76
C-9	Phe16, Met20, Val25, Asp76	Phe16, Met20, Val25, Asp76
C-10	Met20, Thr29	Met20, Glu30
C-11	Phe16, Met20, Thr29	Phe16, Met20, Glu30
C-12	Ala33, Ala75	Gly34, Leu77
C-13	Ala33, Phe57	Gly34, Ile59
C-14	Lys58	Lys58
C-15	Pro38	Pro38
C-16	Pro38, Thr53, Ser55	Pro38, Ser55, Thr57

with the locust M-FABP is 54% and 42% based on the cDNA and amino acid sequence, respectively. Moreover, the locust M-FABP has a significantly higher identity with the human M-FABP than with the other studied invertebrate FABPs, the M-FABP of *Schistosoma mansoni* and both midgut FABPs of *Manduca sexta*. This indicates that the muscle FABP type is highly

conserved. The recently solved three-dimensional structure of human M-FABP [9] and the alignment with the amino acid sequence of locust M-FABP (Fig.3) show that the residues involved in fatty acid binding in the human protein, are highly conserved in the locust M-FABP (Table 2). In human M-FABP residues Arg126 and Tyr128 form hydrogen bonds with one oxygen of the fatty acid carboxylate, these residues are identical in the locust M-FABP. Moreover Arg106, which interacts with a highly ordered water molecule that is hydrogen bonded with the other oxygen of the carboxylate of the fatty acid, is also identical. In human M-FABP Thr40 hydrogen bonds with the same ordered water molecule; in the locust M-FABP this residue is substituted with a Val. Eight out of the fourteen amino acid residues located in the human M-FABP within 0.45 nm of the fatty acid, that have been proposed to interact with the methylenes of the hydrocarbon chain, namely Phe16, Met20, Val25, Ala33, Pro38, Lys58, Asp76 and Arg126, are identical. The six other side chains within the same distance from the fatty acid are conserved and structurally comparable, as shown in Table 2, except for Thr29 which is replaced by a Glu. The residue Phe57, that has been suggested to act as a lid covering the entry of the cavity where the ligand is bound and is within van der Waals distance from the C-13 of the fatty acid in human M-FABP, is an Ile residue in locust M-FABP.

In the human M-FABP the residue Tyr19 has been proposed to be a target for phosphorylation [9], in an analogous way to the Tyr19 in the adipocyte FABP [16,17]. There is a comparable tyrosine residue in the locust M-FABP. Furthermore, the sequence prior to this amino acid, Asn15-Phe-Asp-Asp18, present in both human adipocyte FABP and M-FABP is highly conserved in the locust M-FABP, the Asp residues (17 and 18) being replaced with two Glu residues.

The high overall sequence similarity and the presence of most of the amino acids involved in the fatty acid binding may indicate that the three-dimensional structure and the ligand binding of locust M-FABP are analogous to human M-FABP.

Expression, purification and characterization of the recombinant locust M-FABP

The 3' RACE cDNA fragment, encoding the complete locust M-FABP, was cloned in pET-3d downstream of the T7 RNA polymerase promoter. The 15 kDa locust M-FABP was highly expressed in *E. coli* upon induction with isopropyl ß-D-thiogalactopyranoside and was not detected in uninduced cells. This was verified by SDS-PAGE and Western blotting (results not shown). Recombinant locust M-FABP could be isolated from bacterial lysate by precipitation with 50% ammonium sulphate, delipidation, anion-exchange and gel filtration chromatography (Fig. 4). The yield was 26 mg/l culture. Analysis by SDS-PAGE revealed that the molecular mass of the recombinant protein is identical to the one of the protein isolated from locust flight muscle. The amino acid analysis of the purified protein revealed the quantities of specific amino acids expected on base of the cDNA sequence. In contrast to the protein isolated from locust muscle, the recombinant protein contains the N-terminal methionine. This amino acid seems not to be removed by bacterial processing. Despite their high overall amino acid similarity locust and human M-FABP show no immunological cross-reactivity, indicating

that the amino acids located on the surface of both proteins differ (Fig 5). Both M-FABPs do not react with anti-(human liver FABP) antiserum and human liver FABP does not react with all anti-(M-FABP) antisera (data not shown).

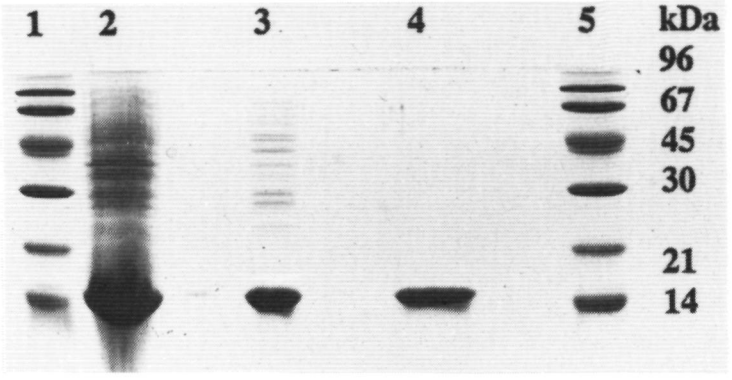

Fig. 4 SDS-PAGE at different stages of purification of recombinant locust M-FABP. Lanes 1 and 5, molecular mass markers (kDa); lane 2, bacterial lysate 4 h after induction (125 μg protein); lane 3, supernatant after 50% ammonium sulphate precipitation (50 μg); lane 4 purified locust M-FABP (15 μg). The proteins were stained with Coomassie Brilliant Blue.

We established binding characteristics of locust M-FABP with oleic acid and various fluorescent ligands and compared these results with new data on human M-FABP. First we determined the apparent K_d values for [1-^{14}C]oleic acid with the Lipidex procedure. The K_d value for recombinant locust and human M-FABP are 0.47 $\pm$ 0.16 μM and 0.43 $\pm$ 0.03 μM (mean $\pm$ SD of 5 independent experiments), respectively. The latter value agrees with previous data on isolated and recombinant human M-FABP [6,8]. The K_d value for the isolated locust M-FABP was also assayed and now established to be 0.59 $\pm$ 0.06 μM (mean $\pm$ SD of 3 experiments). All K_d values found for recombinant and native locust M-FABP are similar, but lower than previously reported [22].

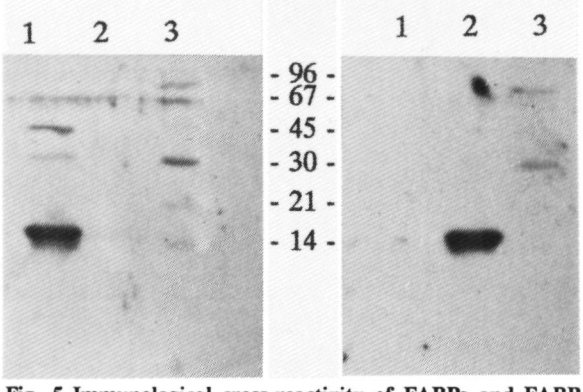

Fig. 5 Immunological cross-reactivity of FABPs and FABP-antisera. Preparations of recombinant M-FABPs (5 μg) were run on SDS-PAGE, transferred to nitrocellulose and immunostained with a polyclonal antiserum against human M-FABP (panel A) and a monoclonal antiserum against locust M-FABP (panel B). Lane 1, human M-FABP; lane 2, locust M-FABP and lane 3, molecular mass markers. A polyclonal rabbit anti-(locust M-FABP) antiserum also only reacted with locust M-FABP (not

With titration microcalorimetry various parameters related to the binding of oleic acid to recombinant locust M-FABP were determined. The locust M-FABP solution was injected into [9,10-^{3}H]oleic acid solution in the sample cell in contrast to previous investigations whereby the fatty acid was injected [43,44]. With our procedure the real concentration of oleic acid present could be determined after the experiment. The loss of fatty acid sticking to the equipment used during the experiment is approximately 60% but does not affect the experiment in this case. The results of a typical titration experiment of oleic acid with locust M-FABP are shown in Fig. 6.

The data were fitted to a mathematical model that assumes one class of binding sites and revealed a molar binding stoichiometry of 1.03 ± 0.13 and a K_d of 0.47 ± 0.08 μM (n=3). This K_d value is in excellent accordance with the value determined with the Lipidex procedure for locust M-FABP. The calculated ΔG, ΔH and ΔS value were -36.1 ± 0.4 kJ/mol, -146 ± 7.5 kJ/mol and 369 ± 26 J/mol K, respectively.

The obtained ΔG value is in good agreement with the ΔG values for fatty acid binding on rat intestinal FABP [43,44] and liver FABP [44] (Table 3). However the ΔH and ΔS values differ markedly from the values found for intestinal FABP [43,44] and liver FABP [44]. These differences may in part be due to the effect of the high fatty acid concentration and the methanol as used in these latter experiments. A potential problem using fatty acid as the titrant is the locally high concentration which will lead to the formation of acid-soaps or micelles. After injection the dissociation of micelles into monomers might affect the thermodynamic parameters. Methanol has been claimed to give a carrier signal, that increases the signal-noise ratio in the measurements [44]. However it also increases the amount of fatty acid in solution, and the real fatty acid concentration was not determined. Moreover the interactions of the methanol with the protein may affect the conformation of the protein and/or

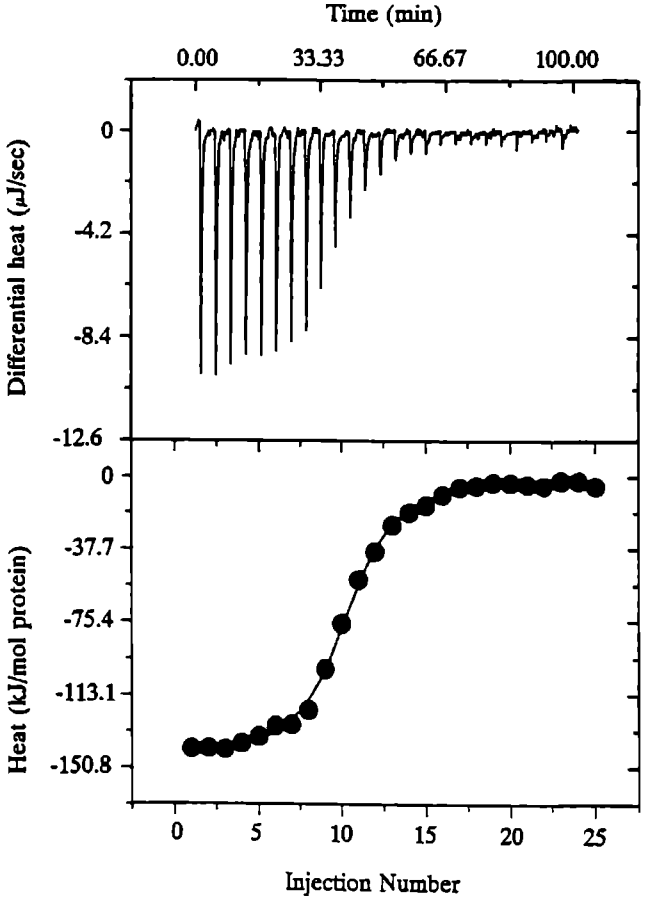

Fig. 6 Titration calorimetry data for the binding of oleic acid to locust M-FABP. The upper panel illustrates the data in the form of an injection diagram. In the lower panel, the filled circles represent the normalized areas under each injection spike. The curve was mathematically fitted assuming one affinity class of binding sites. The fatty acid concentration in the sample cell was between 15 and 25 μM for different experiments, and 25 4-μl-portions of a 0.55 mM locust M-FABP solution were injected. The figure is representative for one out of three experiments.

its binding characteristics. It has been shown for the crystal structure of human M-FABP that ordered solvent closely interacts with bound fatty acid [9]. These two combined effects could have led to an inadequate estimation of the enthalpy and entropy values for intestinal and liver FABP.

Table 3 Fluorescence increase of different fatty acids in reaction with human or locust M-FABP. Experiments were performed with 2.8 μM human M-FABP or 4.0 μM locust M-FABP and 1.0 μM fatty acid. The fluorescence increase is the ratio of the fluorescence with and without protein. Fluorescence increase of 11-dansylamino-undecanoic acid is relative to the emission without protein at 545 nm (excitation at 328 nm), since at 505 nm (excitation 345 nm) no fluorescence without protein is observed. Values are means ± S.D. of three independent experiments.

	Wavelength		Fluorescence increase	
Ligand	excitation nm	emission nm	human M-FABP	locust M-FABP
11-Dansylamino-undecanoic acid	340	505	3.4 ± 0.2	5.0 ± 0.1
2-(9-Anthroyloxy) palmitic acid	365	452	13.3 ± 0 2	8.8 ± 0.2
16-(9-Anthroyloxy) oleic acid	365	452	5.8 ± 0.4	7.9 ± 0.1
16-(9-Anthroyloxy) palmitic acid	365	452	2.5 ± 0 6	1.5 ± 0.3
Cis-parinaric acid	306	410	5.7 ± 0.4	7.9 ± 0.6
Trans-parinaric acid	306	410	5 5 ± 0.5	9 2 ± 0.3

Table 4 The K_i values for various fatty acids with human and locust M-FABP. The K_i values were derived from data of competitive inhibition of [^{14}C]oleic acid binding determined with the Lipidex procedure. To 0.28 μM human M-FABP or 0.4 μM locust M-FABP, equal amounts (1 μM) of [1-^{14}C]oleic acid and competitor were added. The K_i values were determined with displacement analysis as described in Materials and Methods. Values are means ± S.D. of at least three experiments.

Competitor	Human M-FABP μM	Locust M-FABP μM
Palmitic acid	1.11 ± 0.37	0.49 ± 0.06
Linoleic acid	0 51 ± 0.09	1.15 ± 0.41
Linolenic acid	0.48 ± 0.10	1.08 ± 0.20
Archidonic acid	0.95 ± 0.35	2.56 ± 0.38
Cis-parinaric acid	1.26 ± 0.23	1.32 ± 0.35
Trans-parinaric acid	0.49 ± 0 03	0.58 ± 0.05
11-Dansylamino-undecanoic acid	1.11 ± 0.18	0 90 ± 0.19
2-(9-Anthroyloxy) palmitic acid	1.36 ± 0.33	2.12 ± 1.06
12-(9-Anthroyloxy) oleic acid	0.80 ± 0.07	1.25 ± 0.48
16-(9-Anthroyloxy) palmitic acid	0.83 ± 0.08	0 81 ± 0 05

Fluorescent probes were used to analyze the relative fluorescence increase upon binding of fatty acid by recombinant locust and human M-FABP (Table 3). The muscle type FABPs

react similarly with the fluorescent probes and give a markedly lower fluorescence increase than human liver FABP [45]. This large difference in fluorescence increase is due to the more hydrophobic binding site of the liver FABP [45-47]. The differences between the fluorescence increase of both muscle types with 2-(9-anthroyloxy)palmitic acid and 16-(9-anthroyloxy)palmitic acid, 13.3 and 2.5, respectively for human M-FABP and 8.8 and 1.5, respectively for locust M-FABP, in spite of its higher concentration suggest a weaker interaction of locust M-FABP with the fluorescent reporter group.

The low fluorescence enhancement does not allow determination of an apparent K_d value of the fluorescent fatty acids with muscle FABPs. Moreover this parameter may indicate only the interaction of the reporter group with the protein for 11-dansylamino-undecanoic acid and the anthroyloxy fatty acids. We used another method, the displacement of [^{14}C]oleic acid in combination with the Lipidex procedure to determine the K_i values of these fatty acids and of the parinaric acids. The same procedure we applied for four natural long-chain fatty acids (Table 4). In comparison to human M-FABP, the locust M-FABP appears to have a lower K_i value for palmitic acid and a higher K_i value for the C-18 unsaturated fatty acids and especially for arachidonic acid. This may be due to the substitution of Phe57 by Ile59, but also to other differences of the binding cavity. Both M-FABPs have a higher affinity to trans-parinaric than to cis-parinaric acid, in contrast with recombinant rat liver and intestinal FABP [48]. The fatty acids with the anthroyloxy group at the C-2- and C-12-position of the carbon chain show a slightly higher affinity with human M-FABP than with locust M-FABP. Others reported comparable apparent K_d values for the binding of anthroyloxy fatty acids to rodent adipocyte and heart FABPs (17, 49, 50), molecules with a similar tertiary structure as human M-FABP. Generally, the interactions of all fatty acids with both M-FABP types are rather similar, indicating that the locations where the fatty acids bind present the same overall characteristics.

Crystallization and preliminary X-ray diffraction studies of recombinant locust M-FABP

Crystals of locust M-FABP diffract to a maximum resolution of about 0.22 nm. Analysis of the data revealed a primitive trigonal unit cell with dimensions of a=b=5.89 nm, c=14.42 nm and α=90°, β=90°, γ=120°. The volume of the unit cell is 4.33 x 10^4 nm^3. A total of 23,729 reflections over 27,423 possible within the resolution range from 2.0-0.22 nm were collected from a single crystal. The data set showed an R_{merge} based on intensity of 10.2%, a 86.5% completeness in the considered resolution range and a 6-fold redundancy. Further examination of the unreduced diffraction data revealed the absence of all reflections along the reciprocal c axis except the ones with Miller indices (0, 0, 3n). The inspection of the pseudo-precession pictures obtained from the data reduced with a P3 symmetry indicated the presence of a three-fold symmetry axis along c. This finding indicates that the space group is $P3_121$ ($P3_221$). Since there are 6 asymmetric units in the $P3_121$ unit cell, the total volume of the asymmetric unit is 7.2 x 10^3 nm^3. Assuming the presence of two protein molecules per asymmetric unit and a molecular mass of the locust M-FABP of 15 kDa, the volume per unit molecular weight is 0.24 nm^3/Dalton, which is a value in the range expected for a protein

crystal [51]. The high amino acid sequence similarity should allow us to use the human M-FABP as a starting model for the determination of the tertiary structure of the locust M-FABP using molecular replacement methods [52].

In conclusion, the structure of M-FABP is highly conserved during evolution. The primary structure of locust M-FABP shows a high similarity with that of human M-FABP. The conservation of the amino acids relevant for fatty acid binding is reflected in the similar binding characteristics. No immunological relation is however present. Elucidation of the three-dimensional structure will give more information about the position of the relevant amino acid residues.

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CHAPTER 7

SURVEY AND SUMMARY/SAMENVATTING

Many organs of all kinds of species utilize fatty acids as their main fuel. Fatty acids form also important compounds of membrane components as phospho- and glycerosphingolipids. In addition, fatty acids and their Coenzyme A esters may influence directly or indirectly various cellular processes by interference with cell signaling, gene regulation, membrane transport systems or enzyme actions. Fatty acid-binding proteins (FABPs) are supposed to be involved in the intracellular transport of fatty acids and the modulation of fatty acid concentration. These molecules have a low molecular mass (15 kDa) and are present in the cytoplasm. The existence of at least seven FABP types with differences in tissue and cellular distribution and in physicochemical and binding properties is of large interest with respect to their function.

We especially performed our studies on FABPs of kidney since the various parts of the nephron show a marked biochemical and physiological heterogeneity. Marked differences are present in the permeability and transport properties, the oxygen consumption and dependence, the excretion of and sensibility for drugs and the fatty acid metabolism of the nephron segments.

An additional comparative study was performed on the primary structure and binding properties of muscle FABP of *Locusta migratoria* and human to study evolutionary aspects of this protein.

In Chapter 2 an overview is given on the structural and functional aspects of the seven FABP types isolated thus far. Data are presented on their cellular and subcellular distribution. Structural data on the FABP types and on the related members of the FABP family, the cellular retinol- and cellular retinoic acid-binding proteins are compared. The variations in the three-dimensional structure and the protein-fatty acid interaction are discussed together with the methods applied to obtain the information. Variations in ligand specificity and stoichiometry are observed for the different FABP types. The function of FABPs as transfer proteins and their possible modulatory role in various cellular processes as cell signaling, gene regulation, ion transport and lipid metabolism are considered. The gene structure of the members of the FABP family is compared. The regulation of the FABP expression in various conditions has become of interest since in the upstream 5' flanking region of the various FABP genes several sequence elements have been identified that influence the expression level.

In our studies on renal FABPs we started with the purification of the protein from human kidney (Chapter 3). Using ion-exchange and gel filtration chromatography we isolated two FABP types. Characterization based on molecular mass, isoelectric point, fluorescence with DAUDA and immunological crossreactivity showed that type A had much in common with the liver FABP type. The other, type B, was highly identical with the heart/muscle FABP type. Type A, with a molecular mass of 14.5 kDa and a pI of 6.8 showed a fluorescence enhancement and a emission wavelength shift in reaction with DAUDA. Type B, with a molecular mass of 15.5 kDa and a pI of 5.2 showed only a slight fluorescence enhancement and no wavelength shift with DAUDA. Type A is present in the proximal tubules whereas type B is predominantly present in the distal tubules of man. The presence of two FABP types within kidney with a different localization was not described before. In intestine two FABP types, the liver and the

intestinal type, were found but with a similar localization, in stomach four FABP types appeared to be present with a different cellular distribution.

Definitive evidence of the primary structure of the two FABP types present in human kidney was obtained by elucidation of the cDNA sequence (Chapter 4). Based on the protein data we used primers deduced from the liver and heart FABP cDNA sequence in a reverse transcriptase PCR. The cDNAs isolated revealed that type A and B from human kidney are identical with the liver and heart FABP type, respectively. Results with enzyme-linked immunosorbent assay and ion-exchange chromatography suggested that the liver FABP is also present in rat kidney. Using reverse transcriptase PCR we showed that rat kidney also contains both FABP types. Immunolocalization showed that the heart FABP type is similarly distributed in rat kidney as in human kidney. The liver FABP type however is not only present in the proximal tubules but also in the distal and collecting tubules of the rat but its concentration is much less than in human kidney.

The presence of two FABP types in kidney and their different localization suggest a type-specific function. Binding studies indicated that the liver FABP type is a more general carrier and might be involved in excretion processes. The heart FABP type only binds fatty acids and seems to be involved in lipid metabolism. To gain more insight in their possible role, both proteins were expressed in *E. coli* and their binding affinities determined for various hydrophobic ligands (Chapter 5). We obtained efficient expression of human liver FABP in *E. coli*. However the expression was low in comparison with the heart/muscle FABP type and required overnight induction in the presence of the bacterial RNA polymerase inhibitor, rifampicin. The binding characteristics of delipidated recombinant liver FABP and muscle FABP for fatty acids of different chain-length and saturation grade and for various hydrophobic ligands were determined by radiochemical analysis and for liver FABP also by fluorescence. The apparent binding affinity of the ligands was calculated by using displacement curves of oleic acid and DAUDA. Liver FABP showed a preference for binding of long-chain saturated and unsaturated fatty acids up to $C_{24:1}$ whereas the muscle FABP has a preference for unsaturated fatty acids, especially with 18 C atoms. Liver FABP also binds palmitoyl derivatives and other hydrophobic ligands as prostaglandins, 1,25 dihydroxy vitamin D_3, steroid hormone, retinoids and hexadecanedioic acid, generally however with a lower affinity than fatty acids. Muscle FABP only binds fatty acids, testosterone and oestradiol with high affinity. Fatty acids with fluorescent reporter groups are also more tightly bound by liver FABP. A direct assay and displacement studies of oleic acid gave the same K_d value of DAUDA for liver FABP. Fluorescence enhancement and displacement studies indicate that the binding of fluorescent fatty acids is presumably determined by a different kind and location of interaction with both the fluorescent reporter group and the acyl carbon chain (Chapter 5).

In conclusion we established, based on protein and cDNA data, the presence of both the liver and heart FABP type in the kidney. The distribution along the nephron suggests that the liver FABP type, predominantly present in the proximal part, is a more general carrier involved in transport and excretion of all kinds of hydrophobic ligands. The heart FABP type, predomi-

nantly present in the distal part only binds fatty acids and is involved in lipid metabolism. For the continuation of this research, proximal tubule cells cultured on filters offer a good model to study the function of liver FABP in kidney. It is of high relevance to check if nephrotoxic agents are bound by the liver FABP type and really transported in the intact cell. The effect of transfection of those cells with liver FABP cDNA or antisense RNA could give more information on the role of this protein in uptake, transfer and excretion of various ligands. The expression of liver FABP in the cultured proximal tubule cells in the absence or presence of various fatty acids might give information about possible direct regulation of the FABP gene by these ligands.

In an additional project we did a comparative analysis on flight muscle FABP of *Locusta migratoria* and human muscle FABP (Chapter 6). A full-length cDNA of locust muscle FABP was isolated by 3' and 5' rapid amplification of cDNA ends. By comparison of the primary structure of Lm and human muscle FABPs we obtained information about identical and functionally relevant amino acids. The primary structure of muscle FABP appeared to be highly conserved during evolution. The locust muscle FABP was expressed in *E. coli* and analyzed for its immunological behaviour and binding properties. Remarkable was the low immonological cross-reactivity with human muscle FABP in spite of the high similarity. The binding properties were studied by radiochemical displacement assays, titration microcalorimetry and binding assays of fluorescent fatty acids. Generally, the binding characteristics for oleic acid and fluorescent fatty acids of human and locust muscle FABP were similar indicating that the cavities where the fatty acid binds present the same overall characteristics. The recombinant locust protein was crystallized and analysis of the three-dimensional structure was started. The results of this analysis will give additional information to the known three-dimensional structure of human muscle FABP and data obtained in studies on site-directed mutants of this protein.

Functional analysis of muscle FABP is complicated since transport of fatty acids from plasma membrane to cellular organelles cannot adequately be measured or visualized with fluorescent or radiochemical markers. A possibility to determine the function of muscle FABP is to transfect muscle cells with muscle FABP cDNA or antisense RNA or to inactivate the FABP gene by homologous recombination and generate mice which are deficient or partially deficient for this protein.

SAMENVATTING

Veel organen van verschillende diersoorten benutten vetzuren als hoofdbron van energie. Vetzuren zijn ook belangrijke verbindingen van membraan bestanddelen als fosfolipiden en sfingoglycolipiden. Bovendien kunnen vetzuren en hun coenzym A esters direct of indirect verscheidene cellulaire processen beinvloeden door interferentie met signaal transductie, genregulatie, membraantransport systemen of enzymactiviteiten. Vetzuurbindende eiwitten worden verondersteld betrokken te zijn bij het intracellulair transport van vetzuren en de modulering van de vetzuur concentratie. Deze moleculen hebben een laag moluculair gewicht (15 kDa) en zijn aanwezig in het cytoplasma. Het bestaan van minstens zeven FABP typen met verschillen in weefsel en cellulaire verspreiding en in fysisch-chemische en bindingseigenschappen is van groot belang met betrekking tot hun functie.

Wij hebben in het bijzonder onze studies uitgevoerd aan FABPs uit de nier omdat de verscheidene delen van het nefron een duidelijke biochemische en fysiologische heterogeniteit vertonen. Duidelijke verschillen zijn aanwezig in de permeabiliteit en transporteigenschappen, het zuurstofverbruik en -afhankelijkheid, de uitscheiding van en gevoeligheid voor medicijnen en het vetzuurmetabolisme van de nefron segmenten. Bovendien werd een vergelijkende studie uitgevoerd aan de primaire structuur en bindingseigenschappen van spier FABP van Locusta migratoria en mens om de evolutionaire aspecten van dit eiwit te bestuderen.

In hoofdstuk 2 wordt een overzicht gegeven van de structurele en functionele aspecten van de zeven FABP typen die tot dusver zijn geisoleerd. Hun cellulaire en subcellulaire verspreiding wordt besproken. Structurele gegevens van de FABP typen en verwante leden van de FABP-familie, de cellulaire retinol- en cellulaire retinolzuur-bindende eiwitten worden vergeleken. De variatie in de driedimensionale structuur en de eiwit-vetzuur interactie worden bediscusieerd samen met de methoden die gebruikt zijn om deze informatie te verkrijgen. Verschillen in ligandspecificiteit en -stoichiometrie zijn waargenomen voor de verschillende FABP typen. De functie van FABPs als transport eiwitten en hun mogelijk modulerende rol bij verscheidene cellulaire processen als signaaltransductie, genregulatie, iontransport en lipidenmetabolisme worden beschouwd. De genstructuur van de leden van de FABP familie is vergeleken. De regulatie van de FABP expressie onder verschillende omstandigheden is blijkt van belang omdat in de 5' regio stroomopwaarts van de verscheidene FABP genen meerdere DNA elementen zijn geidentificeerd die het expressie niveau beinvloeden.

In onze studies aan nier FABPs zijn we gestart met de zuivering van het eiwit uit humane nier (Hoofdstuk 3). Met behulp van ionenwisseling chromatografie en gelfiltratie hebben we twee FABP typen geisoleerd. Karakterisering op basis van het molecuulgewicht, isoelectrisch punt, fluorescentie met 11-dansylamino-undecaanzuur (DAUDA) en immunologische kruisreactiviteit toonden aan dat type A veel overeenkomsten heeft met het lever FABP type. Het andere, type B, was zeer identiek met het hart/spier FABP type. Type A, met een molecuulgewicht van 14,5 kDa en een isoelectrisch punt van 6,8 toonde een fluorescentie toename en een emissie golflengte verschuiving bij interactie met DAUDA. Type B, met een

molecuulgewicht van 15,5 kDa en een isoelectrisch punt van 5.2 toonde een geringe fluorescentie toename en geen emissie golflengte verschuiving met DAUDA. Type A is aanwezig in de proximale tubuli van de mens terwijl type B voornamelijk aanwezig is in de distale tubuli. De aanwezigheid van twee FABP typen in één orgaan met een verschillende localisatie was nog niet eerder beschreven. In darm werden twee FABP typen, het lever en het darm type, gevonden met eenzelfde localisatie.

Het definitieve bewijs voor de primaire structuur van de twee FABP typen aanwezig in humane nier werd verkregen door opheldering van de cDNA nucleotidenvolgorde (Hoofdstuk 4). Gebaseerd on de eiwitgegevens werden primers, afgeleid van het lever en hart FABP cDNA, gebruikt in een reverse transcriptase polymerase ketting reactie. De nucleotidenvolgorde van de geisoleerde cDNAs toonden aan dat type A en B uit humane nier hetzelfde zijn als het lever en hart FABP type, respectievelijk. Resultaten verkregen uit ELISA en ionenwisseling chromatografie suggereerden dat het lever FABP ook aanwezig is in de nier van rat. Met behulp van de reverse transcriptase polymerase ketting reactie toonden we aan dat rat nier ook beide typen bevat. Immunohistochemische analyse toonde aan dat het hart FABP type op dezelfde wijze verspreid is in rattenier als humane nier. Echter het lever FABP type is niet alleen aanwezig in de proximale tubuli van de rat maar ook in de distale tubuli en verzamelbuizen maar de concentratie is veel lager dan in humane nier.

De aanwezigheid van twee FABP typen in nier en hun verschillende localisatie suggereert een type specifieke functie. Bindingsstudies toonden aan dat het lever FABP type meerdere soorten van liganden bindt en betrokken zou kunnen zijn in uitscheidingsprocessen. Het hart FABP type bindt bijna uitsluitend vetzuren en lijkt betrokken bij het lipiden metabolisme. Om meer inzicht te krijgen in deze mogelijke rol werden beide eiwitten tot expressie gebracht in Escherichia coli en werd de bindingsaffiniteit voor verscheidene hydrofobe liganden bepaald (Hoofdstuk 5). We bewerkstelligden een efficiente expressie van humaan lever FABP in E.coli. De expressie was echter laag in vergelijking met hart/spier FABP en vereiste overnacht inductie in aanwezigheid van de bacteriële RNA polymerase remmer, rifampicine. De bindingskarakteristieken van gedelipideerd recombinant lever FABP en spier FABP voor vetzuren met een verschillende ketenlengte en verzadigingsgraad en voor verscheidene hydrofobe liganden werden bepaald via radiochemische analyse en voor lever FABP ook met behulp van fluorescentie. De bindingsaffiniteit voor de liganden werd berekend met behulp van verdringingscurves met oliezuur en DAUDA. Lever FABP toonde een een voorkeur in binding van langketen, verzadigde en overzadigde, vetzuren tot C24:1 toe terwijl het spier FABP een voorkeur heeft voor onverzadigde vetzuren in het bijzonder met 18 C atomen. Lever FABP bindt ook palmitoyl derivaten en andere hydrofobe liganden zoals prostaglandines, 1,25 dihydroxy-vitamine D3, testosteron, retinoiden en hexadecaandicarbonzuur, echter in het algemeen met lagere affiniteit dan vetzuren. Spier FABP bindt alleen vetzuren, testosteron en oestradiol met hoge affiniteit. Vetzuren met fluorescente reporter groepen worden ook beter door lever FABP gebonden. Een directe analyse en verdringingsstudies met oliezuur leverden een zelfde dissociatieconstante van DAUDA voor lever FABP op. Fluorescentie toename en verdringingsstudies geven aan dat de

binding van fluorescente vetzuren waarschijnlijk wordt bepaald door verschillen in plaats en type van interactie tussen de fluorescente reporter groep en de acyl koolstofketen (Hoofdstuk 5).

Samengevat, we toonden de aanwezigheid van zowel lever als hart FABP type aan in de nier op basis van eiwit en cDNA studies. Hun verdeling over het nefron suggereert dat het lever FABP, voornamelijk aanwezig in het proximale deel, betrokken is bij transport en uitscheiding van allerlei hydrofobe liganden. Het hart FABP type, voornamelijk aanwezig in het distale deel, is betrokken bij het lipiden metabolisme.

In een ander project hebben we een vergelijkende analyse uitgevoerd aan vliegspier FABP van Locusta migratoria en humaan spier FABP (Hoofdstuk 6). Een volledige cDNA van locust spier FABP werd geisoleerd via 3' en 5' snelle amplificering van cDNA einden. Door vergelijking van de primaire structuur van locust en humaan spier FABP verkregen we informatie over identieke en functioneel relevante aminozuren. De primaire structuur van spier FABP bleek zeer sterk geconserveerd tijdens de evolutie. Het locust spier FABP werd tot expressie gebracht in E.coli en het immunologische gedrag werd geanalyseerd. Opvallend was de lage immunologische kruisreactiviteit met humaan spier FABP in tegenstelling tot de grote overeenkomst in primaire structuur. De bindingseigenschappen werden bestudeerd via radiochemische verdringingsstudies, titratie microcalorimetrie en bindings analysen met fluorescente vetzuren. In het algemeen was er een grote overeenkomst tussen de bindingskarakteristieken voor oliezuur en fluorescente vetzuren van humaan en locust spier FABP, hetgeen aangeeft dat de holte waarin het vetzuur bindt een zelfde structuur vertoont. Het locust spier FABP werd gekristaliseerd en opheldering van de drie-dimensionale structuur werd begonnen. De resultaten van deze analyse zullen aanvullende informatie geven bij de bekende structuur van het humaan spier FABP en gegevens verkregen in studies aan mutanten van dit eiwit.

LIST OF PUBLICATIONS

J.H. Veerkamp, R.J.A. Paulussen, R.A. Peeters, R.G.H.J. Maatman, H.T.B. van Moerkerk and T.H.M.S.M. van Kuppevelt (1990) Detection, tissue distribution and (sub)cellular localization of fatty acid-binding protein types **98**, 11-18

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R.G.H.J. Maatman, H.T.B. van Moerkerk, I.M.A. Nooren, E.J.J. van Zoelen and J.H. Veerkamp Expression of human liver fatty acid-binding protein in *Escherichia coli* and comparative analysis of its binding characteristics with muscle fatty acid-binding protein. Biochim. Biophys. Acta. submitted

R.G.H.J. Maatman, M.E. Degano, H.T.B. van Moerkerk, W.J.A. van Marrewijk, D.J. van der Horst, J.C. Sacchettini and J.H. Veerkamp Structure and binding characteristics of an locust and human muscle fatty acid-binding protein. Eur. J. Biochem. submitted

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CURRICULUM VITAE

Ronald Maatman werd geboren op 30 maart 1963 te Raalte. Na het behalen van het VWO-diploma in 1982 aan het Ludger college te Doetinchem werd een aanvang gemaakt met een studie biologie aan de Katholieke Universiteit Nijmegen. Het propedeutisch examen werd gehaald in september 1983 en het docteraal examen met als hoofdvak Microbiologie (12 mnd., Prof. dr. Ir. G. Vogels) en bijvakken Biochemie (6 mnd., Dr. W. de Grip) en Moleculaire Genetica (9 mnd., Dr. A. Berns) in september 1988. Tijdens zijn docteraal studie assisteerde hij bij het microbiologisch practicum.

Vanaf 1 november 1988 tot 1 november 1992 was hij als assistent in opleiding in dienst bij de afdeling Biochemie, Faculteit van Medische Wetenschappen aan de Katholieke Universiteit Nijmegen. Gedurende deze periode verrichtte hij onder supervisie van Prof. Dr. J.H. Veerkamp het in dit proefschrift beschreven onderzoek. Tevens werden studenten Biologie en Scheikunde en studenten van het HLO tijdens hun stage begeleid en leverde hij een bijdrage aan het onderwijs aan medische studenten.

In september 1989 en 1992 bezocht hij the the first and second International Workshop on Fatty Acid-Binding Proteins en in september 1990 the 31 st International Conference on the Biochemistry of Lipids (ICBL). In juli 1992 hield hij de lezing: Fatty acid-binding proteins: structural and functional diversity op de 643 rd Biochemical Society Meeting aan de Universityof Warwick. In maart 1993 bracht hij een werkbezoek aan de afdeling biochemie van Prof. Dr. J.C. Sacchettini op het Albert Einstein College in New York.

Sinds 1 augustus 1993 is hij als post-doc in dienst op de afdeling van Dr. Achim Gossler op het Max Planck instituut in Keulen.

Op 19 mei 1993 trouwde hij met Mirjam van Zuylen.

In 1987, 1988, 1989 en 1992 was hij met zijn ploeggenoten van Atletiek Vereniging Nijmegen nationaal kampioen op de 4x800 m estafette en is sinds 1988 ook nationaal recordhouder op deze afstand.